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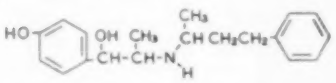
PRODUCTS RECENTLY EVALUATED BY THE A. M. A. COUNCIL ON PHARMACY AND CHEMISTRY

NYLIDRIN HYDROCHLORIDE.—Arlidin (Arlington Funk).—$C_{18}H_{23}NO_2 \cdot HCl$.—M.W. 345.88.—1-(*p* - hydroxyphenyl) - 2 - (1 - methyl - 3 - phenylpropylamino)propanol hydrochloride.—The structural formula of nylidrin hydrochloride may be represented as follows:



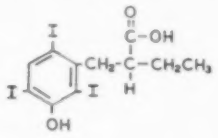
Actions and Uses.—Nylidrin hydrochloride is an epinephrine-ephedrine type of compound that acts as a peripheral vasodilator. The site of action is the same as that of other sympathomimetic drugs, but its effect is vasodilator only, producing relaxation of the muscle fibers in the media of the vessel wall. Its action appears to be almost selective upon the small arteries and arterioles of skeletal muscle. It increases muscle blood flow but has little or no effect upon the superficial skin vessels. This action upon the vessels of skeletal muscle has been demonstrated in both human and animal experiments. Studies with plethysmographic measurements have confirmed the ability of nylidrin hydrochloride to increase muscle blood flow and to exercise tolerance in peripheral vascular disease.

The activity of nylidrin hydrochloride results in the mobilization of depot blood and brings about an increased cardiac output. In the hypertensive individual the mean arterial pressure may drop, whereas in the hypotensive patient the mean arterial pressure may actually increase, in comparison to the sitting normotensive individual in whom the mean arterial pressure is either unaltered or only slightly diminished. The drug is rapidly absorbed from the gastrointestinal tract and is only slightly affected by digestive enzymes. It is slowly metabolized and has a protracted action.

Nylidrin hydrochloride is indicated in vascular disorders of the extremities that may be benefited as the result of increased blood flow. Accordingly, it is worthy of trial in intermittent claudication and Raynaud's disease and as an adjunct to other measures in severe forms of peripheral vascular disease such as diabetic vascular disease, thrombophlebitis, thromboangiitis obliterans, ischemic ulcers, and endarteritis obliterans. Minor side-effects such as nervousness or palpitation may occur, but usually these are transient and disappear with continued therapy or reduction of dosage. The drug should be used with caution in hyperthyroidism, paroxysmal tachycardia, and severe angina pectoris. It is contraindicated in acute coronary thrombosis.

Dosage.—The suggested initial dosage for adults is 6 mg. orally three times daily. This may be increased to four to six times daily, depending upon clinical response and individual tolerance. If side-effects develop and persist, the dose should be reduced to 3 mg. three times daily or therapy temporarily discontinued and then resumed with lower doses.

IOPHENOXIC ACID.—Teridax (Schering).—$C_{11}H_{11}I_3O_3$.—M.W. 571.96.—α-Ethyl-3-hydroxy-2,4,6-triiodohydrocinnamic acid.—α-Ethyl-β-(3-hydroxy-2,4,6-triiodophenyl)propionic acid.—The structural formula may be represented as follows:



Actions and Uses.—Iophenoxic acid is a water-insoluble organic iodine compound for oral administration as a radiopaque agent in cholecystography. It produces a reasonably consistent and dense gallbladder shadow for roentgenographic visualization of nonopaque stones and for observing the functional capacity of the gallbladder to concentrate and then expel bile following a high fat meal. It also permits visualization of the extrahepatic ducts after cholecystography. After oral administration, it is rapidly absorbed, eliminated in the bile, and subsequently excreted, chiefly in the urine. Thus it seldom produces opacities in the gastrointestinal tract. An optimal concentration in the gallbladder usually appears within 8 to 12 hours after ingestion of a single dose; the density of the shadow produced with the recommended dose ordinarily does not obscure stones that may be present.

Iophenoxic acid has a low systemic toxicity and a low level of unpleasant side-reactions. Nausea, vomiting, and diarrhea are minimal, rarely severe. Complaints of a burning sensation while swallowing have not been reported, and subsequent dysuria occurs infrequently. As with other systemically administered iodinated compounds, it should be used with extreme caution, if at all, in patients with advanced renal impairment. The calculated risk involved with its use in other conditions in which iodine may be contraindicated, should be weighed against the need for cholecystographic diagnosis.

Dosage.—Iophenoxic acid is administered orally. The usual dose is calculated on the basis of approximately 50 mg./Kg. (2.2 lb.) of body weight. A dose of 3 Gm. is usually adequate for a patient weighing 68 Kg. (150 lb.); a dose of 6 Gm. should not be exceeded. For cholecystography, the patient is instructed whenever feasible to eat a high fat lunch the day the agent is to be administered to encourage evacuation of the gallbladder so that it may refill with opacified bile; the evening meal should be fat

(*Continued on page IV*)

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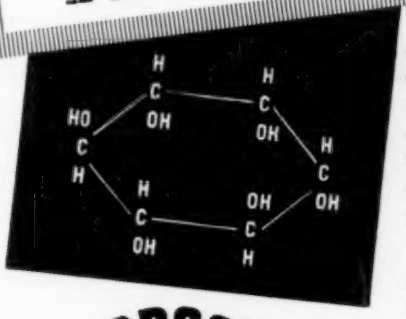

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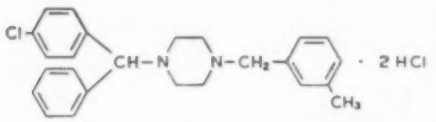
. . . . . *from page II*

free. The calculated dose is taken with a glass of water at such time during the evening as will permit a 10-hour interval to elapse prior to the time scheduled for roentgenography on the following morning. A fatty meal ingested after the cholecystrographic examination usually permits roentgenographic visualization of the extrahepatic ducts 20 to 30 minutes after the meal. If the gallbladder is not visualized following the initial dose and this cannot be attributed to extracholecystic factors, the examination should be repeated the following day with the same dose. Nonvisualization after a second dose usually indicates a pathological condition of the gallbladder.

MECLIZINE HYDROCHLORIDE.—Bonamine HCl (Pfizer).—$C_{25}H_{27}ClN_2.2HCl$.—M.W. 463.87.—1 - (*p* - Chlorobenzhydryl) - 4 - (*m* - methylbenzyl)-piperazine dihydrochloride.—The formula may be represented as follows:



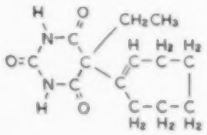
Actions and Uses.—Meclizine hydrochloride, like other antihistaminic agents, depresses the central nervous system. It exhibits a pronounced action against motion sickness as measured in human beings by a depressive effect upon the excitability of labyrinth and vestibular-cerebellar nerves. It is not yet clear whether such action can be explained by the interference with histamine (or a histamine-like substance), by the central depressant effect, or both. Pharmacological studies indicate that it has an antihistaminic potency at least as great as that of other similar agents and a somewhat longer duration of action. The action of a single oral dose is prolonged over a period of 9 to 24 hours.

Meclizine hydrochloride is effective in the prevention or treatment of motion sickness, and in the treatment of nausea and vomiting associated with vertigo, labyrinthitis, Ménière's syndrome, and radiation sickness.

Meclizine hydrochloride, like other antihistamines, may produce drowsiness and other side-reactions, such as blurred vision, dryness of the mouth, and fatigue. Therefore, it should be used with caution by individuals whose activities require constant alertness in the operation of machinery, especially vehicles. Studies so far do not reveal that the drug is any more toxic than other similar agents, but until more is known of its long-term effects, physicians should be on guard for the appearance of untoward reactions. When prolonged use may be indicated, periodic blood cell counts should be made.

Dosage.—Meclizine hydrochloride is administered orally. An average oral dose for adults of 25 to 50 mg. once daily is recommended for the prevention of motion sickness. Ideally, the initial dose should be taken about one hour prior to embarkation to insure absorption (and retention) of the drug prior to the development of symptoms. Children require about one-half the adult dose. Similar oral dosage may be employed as tolerated in the treatment of radiation or motion sickness and for the control of nausea and vomiting in diseases involving the labyrinth or eighth cranial nerve.

HEPTABARBITAL.—Medomin (Geigy).—$C_{13}H_{18}O_3$.—M.W. 222.29.—5-(1-cyclohepten-1-yl)-5-ethylbarbituric acid.—The formula may be represented as follows:



Action and Uses.—Heptabarbital is qualitatively similar in action to other short-acting, chemically related barbiturates. It produces sedation, hypnosis, or anesthesia, depending on the dose administered, but has not been demonstrated to have specific analgesic properties. It exerts a mild spasmolytic effect on smooth muscle and produces mild hypotension as a result of central sedation and peripheral vasodilation. Low grade tolerance develops readily with repeated administration, but this does not appear to be greater than that observed with other barbiturates. Euphoria may accompany the use of fairly large doses, and the drug is potentially capable of inducing habituation and physical dependence.

Heptabarbital appears to be readily absorbed from the gastrointestinal tract and undergoes rapid metabolic destruction in the body. The latter characteristic probably accounts for its short duration of action and relative lack of cumulative effects. Biochemical studies indicate that the compound first is oxidized to a ketone, which then undergoes such rapid further degradation that less than 4% of the ketone can be recovered in the urine; none of the drug is excreted unchanged. Whereas the experimental and some clinical studies demonstrate that heptabarbital accumulation in the body is less than with pentobarbital, this represents only a theoretical clinical advantage and does not take into consideration individual variations in the detoxication capacity of the liver or other organs.

Heptabarbital is worthy of clinical trial as a comparatively short-acting barbiturate in all situations for which mild sedation or hypnotic action is indicated. Its use as an anesthetic agent has not been adequately explored. At the hypnotic dosage level its acute toxicity is about one-third that of pentobarbital, 0.6 Gm. of heptabarbital being roughly equivalent to 0.2 Gm. of pentobarbital. A single hypnotic oral dose of 0.2 to 0.4 Gm. of heptabarbital, given 30 to 45 minutes before retiring, rarely is associated with "hangover." A minimum of side-effects has been observed after daily doses of 0.6 to 2.4 Gm.; gastrointestinal disturbances, skin eruption, excitement, or other side-effects are rarely encountered. Chronic toxicity studies in dogs receiving 100

(*Continued on page VI*)

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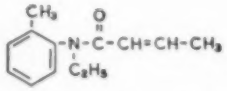
. . . . . *from page IV*

mg./Kg. of body weight per day for 90 days have revealed no unusual biochemical changes and no evidence of injury to the parenchymatous tissues or the blood-forming organs. Until longer clinical experience with the drug indicates otherwise, however, it should be employed with the general precautions applicable to all barbiturates.

Dosage.—Heptabarbital is administered orally. The optimal effective dosage has not been conclusively established. For sedation, 50 to 100 mg. two or three times daily is suggested. The suggested hypnotic dose is 0.2 to 0.4 Gm. before retiring. Some patients may require a hypnotic dose of 0.6 Gm. Until the effective dosage is more firmly established, it may be preferable to start with the suggested lower amounts, adding moderate daily increments until a satisfactory level of sedation or hypnosis is obtained.

CROTAMITON.—Eurax (Geigy).—$C_{13}H_{17}NO$.—M.W. 203.27.—N-Ethyl-*o*-crotonotoluide.—The structural formula of crotamiton may be represented as follows:



Actions and Uses.—Crotamiton is a sarcopticide useful for topical application in the treatment of scabies. Although it exhibits some bacteriostatic and fungistatic properties, there is inadequate evidence at present to justify claims for such action or for use in place of recognized local anti-infective agents when these may be indicated for the management of secondary infection.

Sensitivity or intolerance to crotamiton itself has been rarely encountered; mild erythema observed in some cases has been attributed to its cream base. Although crotamiton apparently has a low index of sensitization, physicians should be aware that such reactions may be encountered. In the presence of acute vesicular dermatitis crotamiton should not be used, for it may produce a burning sensation and act as a primary irritant. Crotamiton is of low toxicity in animals. Systemic effects have not been observed clinically.

Dosage.—Crotamiton is applied topically in a cream or lotion base containing 10% of the agent. In the treatment of scabies, it is applied to the skin by gentle massage in an amount sufficient to cover the entire body (excluding the head); 30 Gm. is usually sufficient for a single application. Preliminary bathing is not necessary, but if this is done the skin should be thoroughly dry before applying crotamiton. A second application 24 hours later is advised to assure complete eradication of the mites; occasionally, a third application may be required. A cleansing bath should be taken 48 hours after the last application and bed and body clothing should be completely changed to prevent reinfection. Other members of the family should be examined for the disease.

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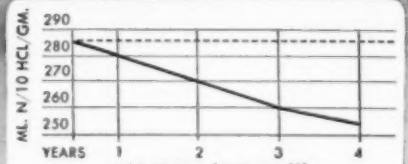

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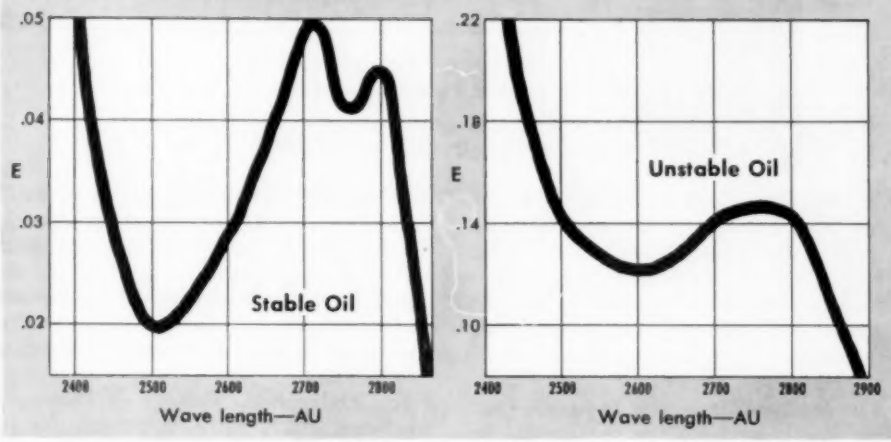

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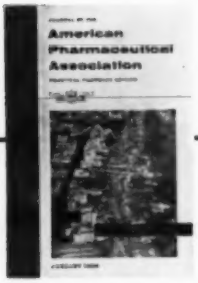

A Preview of Advertising

In the June 1956 issue of the Practical Pharmacy Edition

(All references to actions and uses are taken from the statements of the producers of the products mentioned.)

▸ In its long-familiar, *looked-for* and *looked-at* position on the second cover, **Abbott Laboratories** delineates the "plus" factors of its dietary supplement, **Sur-Bex;** and elsewhere, in a two-page spread, describes the efficacy of its antibiotic **Erythrocin.**

▸ **The American Druggists' Insurance Company** *For Druggists Only* stresses the *fire protection* it offers in its current announcement.

▸ "Just Dip and Read!" That's how **Ames Company** emphasizes the simplicity and convenience with which **Clinistix** *Reagent Strip* can be used to determine the presence of glucose in urine.

▸ **Lactinex** *Granules* and *Tablets,* products of **Hynson, Westcott & Dunning,** are indicated as effective in the treatment of diarrhea.

▸ The phrase, "New instant relief from poison ivy, insect bites," introduces **Lederle's Rhulispray.**

▸ **Mi-Cebrin,** produced by **Eli Lilly and Company,** "tops the nutritional scale" and is advocated "for vigorous maturity."

▸ **Salcort,** offered by **The S. E. Massengill Company,** is described as a "judicious" combination of salicylates and cortisone which results in "a much more favorable reaction in arthritis than with either alone."

▸ A timely announcement is that of **Parke, Davis & Company** pointing up the relief **Caladryl** *Lotion* and *Cream* offer from sunburn, prickly heat, insect bites, and minor skin infections.

▸ In one announcement **Pfizer** discloses that **Terra Bon** offers a *new taste* (peach-flavored, peach-colored) in antibiotic therapy; and in another message, offers the reminder that **Bonamine,** *longest-acting motion-sickness remedy,* is a sales item that goes hand in hand with summer travel.

▸ The **Philadelphia College of Pharmacy and Science** announcement reflects that its courses are preparatory for *careers* in all phases of pharmaceutical endeavor.

▸ Offered by **Pictorial Paper Package Corp.** is a *new* "Moisture Guarded Prescription Package" described as "the most revolutionary" improvement since the introduction of the hinged-lid.

▸ The **Schering** *Library of Medical Films* presents three new color reviews of various aspects of endocrinology: "The Physiology of Normal Menstruation," "The Male Sex Hormone," and "The Menopause: Its Significance and Managements."

▸ **Smith, Kline & French** adds still another affiant to the many testifying to the sales-effectiveness of its **Benzedrex Inhaler** display carton.

▸ *New* from the laboratories of **Warner-Chilcott** is **Sterisil Vaginal Gel,** a new chemotherapeutic agent for treatment of vaginitis.

▸ Advocated by **Winthrop Laboratories** as affording greater response in anemias are *new* **Fergon Plus** *Caplets.*

▸ For a real "eye-opener" **Wyeth** calls attention to its current deal on **Collyrium,** "America's favorite eye lotion," which puts the emphasis on *profit.*

Look for details in the Practical Pharmacy Edition of THIS JOURNAL*—Out June 20*

Scientific Edition

JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION

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The Exposure of Drugs to the Effects of Nuclear Explosions*,†

By EDWIN P. LAUG

Certain drugs were subjected to high levels of ionizing radiation and shock and the effect of nuclear explosions on their potency and toxicity was determined. Induced radioactivity found in some of the drugs was traced to the presence of mineral excipients such as calcium phosphate. All chlorine-containing drugs became radioactive by transmutation of some of the chlorine to sulfur 35. It appears that only a slight radiological health hazard may be involved in the use of exposed drugs under disaster conditions. Despite this, however, special precautions should be taken in the use of drugs exposed to radioactivity if they contain chlorides or phosphates.

AFTER AN ATOM bomb attack on any community there will be an urgent and immediate need for drugs and standard medical preparations to treat the large number of casualties expected, as well as to continue the treatment of persons whose health depends on such substances as insulin. The questions arise whether the drugs and supplies salvaged from drugstores and pharmacies in the zone exposed to the bomb will be so affected by the radiations as to lose their potency or become poisonous, or whether the containers will be so damaged by shock or blast effect as to cause the contents to become unsterile. In an attempt to answer some of these questions the Food and Drug Administration exposed a representative group of drugs and medical preparations to nuclear explosions at the Nevada Test Site in the spring of 1953.

EXPERIMENTAL

Procedure.—Forty-two preparations were exposed. Drugs were selected that were considered essential in critical situations likely to arise after atomic bombing. A complete list was not attempted; it was the purpose rather to study the effects of radiation and blast on a limited representative group of drugs so as to delineate the problems involved and give guidance for further experimentation if it proved necessary.

With few exceptions, all drugs were contained in glass bottles or ampuls, and in the original commercial package units. The size selected was the common one that might be found on the drugstore shelf or moving in commerce. However, the findings on the retail size units were considered applicable also to bulk units.

* Received March 16, 1955, from Division of Pharmacology, Food and Drug Administration, Department of Health, Education, and Welfare, Washington 25, D. C.

† A considerable portion of the work presented in this report represents the efforts of a number of investigators and groups. Their assistance is hereby gratefully acknowledged. Neutron flux measurements: Mr. Eugene Tochilin, U. S. N. Radiological Defense Laboratory, San Francisco, Calif. Gamma flux measurements, film method: Mr. L. J. Deal, Radiation Instruments Branch, Division of Biology and Medicine, U. S. Atomic Energy Commission, Washington, D. C. Gamma flux measurements, chemical method: Mr. Sanford C. Sigoloff, Atomic Energy Project, University of California, Los Angeles, Calif. Assistance in the placement and recovery of the drug boxes at the test sites: Mr. Jack C. Greene, U. S. Civil Defense Administration, Washington, D. C. Chemical and biological data: The Divisions of Antibiotics, Microbiology, Food, Nutrition, Pharmaceutical Chemistry, and Pharmacology, U. S. Food and Drug Administration, Department of Health, Education, and Welfare, Washington, D. C.

This report is a modified version of the report submitted to the director of the Civil Effects Test Group, Operation Upshot-Knothole, Spring 1953.

The drugs were arranged for exposure by packing in heavy wooden boxes.[1] These boxes were 4 inches deep, 18 inches wide, and 25 inches long. The frames were of 7/8-inch stock with 3/4-inch plywood tops and bottoms, all parts secured with 2-inch iron screws. The space in each box was divided into compartments with 1/2-inch plywood slats extending in the longest dimension. The slats served to restrict possible movement of the contents in the event of violent shock. The drug units, in addition to being individually wrapped in paper, were packed in a single layer between liberal amounts of excelsior. Single layer packing was used to reduce shadowing effects. All of the heavy bottles, containing from 250 to 500 ml. of perfusion solutions, were packed in one compartment and tightly wedged between wrappers of corrugated paper. The arrangement of the drug units was identical in all boxes.

In Table I is given the list of drugs and drug preparations that were contained in each box. A total of 20 boxes was prepared and shipped to the test site. Of these, 17 were exposed. The remaining three were never exposed, but were used as controls against possible deterioration or damage to the contents during transit.

Wherever possible, the form of the drug selected for exposure was the form commonly administered. About half of the preparations were, therefore, in powder or tablet form. Many of the tablets contained therapeutically inert additions, such as excipients and stabilizers, which are known to complicate analytical methods. Consequently, in the case of the important antibiotics, pure crystalline forms were also exposed together with the therapeutic forms. The thioglycollate medium was prepared in the laboratory and then sterilized and sealed with standard commercial equipment. This test was intended to detect contamination in case the seal failed momentarily during violent pressure changes.

In order to study possible chemical deterioration of common closure materials such as gum rubber, tygon plastic, and natural black rubber, these materials were reduced to small pieces and enough of each suspended in dextrose saline medium so as to increase the ratio of exposed closure material area to solution about twenty-fold. The resultant preparations were then sterilized and sealed with standard commercial equipment.

Exposures were made at high levels of shock and radiation flux by placing the samples relatively close[1] to the nuclear explosion. Additional exposures were also made at graduated distances farther removed. For the purposes of this report, exposures at the relatively close distance will be referred to as "critical"; those at graduated distances farther removed as "intermediate." No structural shielding was used. The samples were placed in shallow trenches and protected by a thin layer of soil. Experience showed that this procedure prevented gross physical destruction of the samples without significant shielding to radiation flux. While such a procedure could hardly be justified as realistic from the standpoint of the usual way drugs are stored, it was felt that it did simulate the very worst stress situation. Such a situation might occur in a modern steel and concrete structure located on the periphery of the "zone of total destruction" associated with the explosion of a nominal type A bomb.

The drug boxes were instrumented with the following detecting elements: (*a*) neutron detectors consisting of sulfur for fast, and gold for thermal neutrons; (*b*) gamma ray detectors consisting of film, and chemical dosimeters of the chlorinated hydrocarbon type; (*c*) thermal test papers.

[1] While distances, overpressures, and radiation flux referring to the specific nuclear explosions to which the drug samples were exposed are not mentioned in this report, orientation may be made in terms of a nominal type atomic bomb of 20,000 Kt equivalent yield. In terms of this weapon the samples placed "relatively close" were within a radius of 1/2 mile from ground zero. According to "The Effects of Atomic Weapons" publication, a half-mile radius defines an area of about 3/4 mile in which there is virtually complete destruction. By orders of magnitude: blast overpressure, 30–50 p. s. i.; gamma flux, 10,000–20,000 r; slow and fast neutrons, 10^{11} to 10^{13} per $(cm.)^2$.

Methods of Testing Drugs after Exposure

Radioactivity.—Radioactivity induced by high neutron flux in the drugs was first surveyed with standard portable beta-gamma meters at the test site. Later, additional determinations were made with a Geiger counter-scaler standardized against a Bureau of Standards beta source. In order to reduce self absorption, the drug preparations in these latter tests were ashed. Measurements were also repeated at intervals over a four-month period in order to determine roughly the apparent half-life of the induced activity. Efforts were also made to identify the elements in the drug samples chemically and to correlate these findings with the observed apparent half-lives.

Chemical Tests.—Eighteen of the drug preparations were examined in an infrared spectrophotometer. This technique was used as a screening test to decide whether more extensive biological methods would be worth while. It is estimated that this instrument was sensitive enough to record changes of $\pm 10\%$ in composition. Qualitative spectroscopic examination was made on 20 ashed drug preparations in order to establish the presence of the main elemental components as well as trace elements of possible significance.

Biological Tests.—Seven standard bioassay techniques were applied to 13 different drug preparations to establish potency. In addition, a number of other biological tests such as blood pressure, E.C.G., guinea pig intestine, mortality, etc., were applied to a variety of different drugs to test for possible acquired toxicity.

Sterility.—Standard sterility tests were run, as well as bioassays to determine the presence or absence of pyrogens.

Closures.—These were examined for physical intactness, as measured by standard sterility or pyrogenicity tests, and for chemical deterioration, as evidenced by changes in the drug through contact with the closure material.

RESULTS

Radioactivity.—Significant radioactivity as measured with portable beta-gamma survey meters was noted in the drug boxes critically exposed. For example, in one test, readings of 20 to 40 mr/hr. were obtained forty-eight hours after exposure; in an-

TABLE I.—LIST OF DRUGS EXPOSED TO NUCLEAR DETONATIONS

Drug Preparation	Form	Unit	Units per Package
Vitamin D	Gelatin capsule	50,000 u.	500/bottle
Vitamin B_{12}	Glass ampul	10 ml.	1 ampul
Vitamin K	Glass ampul	1 ml.	6/pkg.
Folic acid	Tablet	5 mg.	100/bottle
Meperidine hydrochloride	Tablet	50 mg.	25/bottle
Morphine sulfate	Tablet	15 mg.	20/vial
Pantopon	Tablet	50 mg.	20/vial
Methadone hydrochloride	Glass vial	10 ml.	1 vial
Amyl nitrite perles	Glass capsules	0.3 ml.	12/metal box
Nitroglycerine	Tablet	0.6 mg.	100/bottle
Methantheline bromide	Tablet	50 mg.	100/bottle
Phenobarbital	Tablet	30 mg.	100/bottle
Thiopental sodium	Sterile powder	1 Gm.	6 amp/box
Prantal methyl sulfate	Tablet	100 mg.	100/bottle
Aspirin	Tablet	300 mg.	24/bottle
Atropine sulfate	Tablet	0.4 mg.	100/bottle
Digifolin	Glass ampul	2 ml.	20/box
Epinephrine hydrochloride	Glass ampul	1 ml.	12/box
Sulfadiazine	Tablet	500 mg.	100/bottle
Insulin	Ampul	40 u.	10 ml./amp.
Protamine zinc insulin	Ampul	40 u.	10 ml./amp.
Ethyl ether	Metal canister	1/4 lb.	1 canister
Aureomycin	Capsule	250 mg.	16/vial
Aureomycin	Crystalline powder	2 Gm.	1 vial
Chloramphenicol	Capsule	250 mg.	16/vial
Chloramphenicol	Crystalline powder	2 Gm.	1 vial
Terramycin	Crystalline powder	200 mg.	1 vial
Penicillin, potassium G	Crystalline powder	500,000 u.	1 vial
Penicillin, procaine in oil	Glass vial	300,000 u.	1 vial
Penicillin dibenzyl aq. suspension[a]	Glass bottle	2 fl. oz.	1 bottle
Bacitracin	Powder	1 Gm.	1 vial
Streptomycin	Powder	1 Gm.	1 vial
Cortisone	Powder	50 mg.	1 vial
Isotonic sodium chloride	Glass ampul	100 ml.	1 ampul
Dextrose 10% in water	Glass bottle	250 ml.	1 bottle
Dextran	Glass bottle	500 ml.	1 bottle
Polyvinylpyrrolidone (PVP)	Glass bottle	500 ml.	1 bottle
Benzalkonium chloride	Glass bottle	4 fl. oz.	1 bottle
Thioglycollate medium	Glass bottle	250 ml.	1 bottle
Tygon intravenous tubing	Finely divided	7 Gm./250 ml.	Dextrose-saline 1 bottle
Latex (gum rubber) tubing	Finely divided	9 Gm./250 ml.	Dextrose-saline 1 bottle
Lacquered nat. rubber stoppers, black	Finely divided	20 Gm./250 ml.	Dextrose-saline 1 bottle

[a] Dibenzylethylenediamine dipenicillin G.

other, 400 mr/hr. twenty hours after exposure. Removal of dust from the surface of the boxes, even wiping with a moist cloth, did not materially reduce the activity. Upon opening the boxes it was found that practically all the activity came from the large glass bottles containing perfusion solutions. When the contents were removed, the readings were not materially reduced; this showed that most of the activity resided in the glass. Increasing depths of dry earth shielding inversely affected the activity of the glass bottles, as shown in Table II. When these readings were repeated five days later it was found that the activity had declined to values hardly measurable with survey instruments. Estimates indicated that the decay rate approximated that for sodium 24 which has a half-life of fifteen hours. Since most types of commercial glass contain appreciable quantities of sodium, it may be assumed that the high but rapidly declining activity of the glass is due to sodium 24 formed by an n-gamma reaction from sodium 23.

TABLE II.—EFFECT OF SHIELDING ON THE INDUCED RADIOACTIVITY OF GLASS BOTTLES AND CONTENTS

	Milliroentgens per Hour[a] —2 Days after Exposure— "PVP"	
Depth of Soil Shielding, ft.	Perfusion Bottle, 500 ml. Capacity	Dextrose Bottle, 250 ml. Capacity
2	1.75	1.5
1	11.5	9.7
0	20.0	20.0

[a] Beta-gamma survey meter, tube shield open.

In this connection, the interesting observation was made that all the clear glass located at critical exposure stations exhibited a marked "darkening" without notable loss in transparency. In fact, the degree of darkening was directly proportional to the amount of exposure, as was shown when the glass from other boxes at intermediate distances was compared. After nearly seven months, there is no visible change in the glass, although it was, to be sure, stored out of contact with direct sunlight. *In practical situations, this darkening of glass could serve as a warning of heavy exposure to ionizing radiation.* Fig. 1 shows an example of the phenomenon.

In some of the smaller drug packages containing solid materials, readings were also made with the beta-gamma survey meter on the container and

contents, the empty container, and the contents alone.

The results are shown in Table III. It is clear that most of the activity comes from the glass container, with variations accounted for mainly by the size and thickness of the glass. However, in the case of methantheline bromide, phenobarbital, thiopental sodium, Prantal® methyl sulfate, sulfadiazine, and Aureomycin,® there is no question that significant activity has also been acquired by the drugs themselves.

To gain some idea at what rate the radioactivity of these six drugs decayed, they were re-examined after five days. The results are shown in Table IV. The apparent short half-life of methantheline bromide and thiopental sodium is accounted for chiefly by the presence of bromine 82 and sodium 24 with respective half-lives of thirty-four and fifteen hours. The longest apparent half-life, Prantal methyl sulfate may be attributable to sulfur 35 with a half-life of 87 days. Presumably the presence of other radioactive elements has the effect of reducing the apparent half-lives to mere approximations of the true values. All of the elements shown were not elements of constitution. A notable example was Aureomycin where the presence of phosphorus was due to a calcium phosphate excipient.

Orientation with respect to the significance of these radioactive drugs may be obtained by reference to Table V. Here are tabulated the amounts of radioactivity in microcuries per day which would be ingested from an average dose of the drug when taken two, five, and thirty days after exposure to a nuclear explosion. Under the 4 elements listed with their respective daily tolerance doses: S (significant) and NS (not significant) show whether or not the tolerance would be exceeded. In all examples the tolerance dose for P^{32} would be exceeded if the respective drug were taken at its average daily intake. In the case of this isotope, with a half-life of 14 days, significant radioactivity extends to at least 30 days, while Br^{82}, with a half-life of thirty-four hours, decays so rapidly that its significance vanishes shortly after five days. No example of excessive concentrations of S^{35} was noted. In the case of the Na^{24} species, the small quantities of methantheline-bromide and pentothal sodium given daily do not exceed the tolerance. However, perfusion fluids containing sodium chloride are usually given in relatively large quantities, and there is no question that the tolerance for Na^{24} would be significantly exceeded if such fluids were used within the first week after exposure.

In addition to the above six drugs the others were also tested for radioactivity. Measurements as shown in Table VI were made on ashed samples

TABLE III.—INDUCED RADIOACTIVITY OF DRUG CONTAINERS AND CONTENTS

	Milliroentgens per Hour[a] — 2 Days After Exposure —		
	Container + Contents	Container	Contents
Vitamin D	25–30	25–30	0.5
Folic acid	15	16	0.0
Meperidine hydrochloride	22	22	0.13
Nitroglycerine	13.5	14	0.0
Methantheline bromide	20	18	17.0
Phenobarbital	17.5	19.5	3.0
Thiopental sodium	16.5	...	5.5
Prantal methyl sulfate	16	17	0.9
Aspirin	25–30	25–30	0.13
Sulfadiazine	22	22	0.9
Cortisone	17.5	19	0.0
Aureomycin capsules	22	22	5.0
Chloramphenicol capsules	19.5	20	0.1
Potassium penicillin G	18	17	0.2
Bacitracin powder	6.5	6.5	0.15
Streptomycin	21	20	0.0

[a] Beta-gamma survey meter, tube shield open.

thirty days after the exposure. Activities, expressed as microcuries per gram of drug preparation and microcuries per average daily intake, are given in columns 3 and 4. By reference to column 2, it is possible to gain some insight as to the nature of the excipient. This column shows the ratio of ashed to unashed drug. For example, an organic excipient such as lactose (atropine) gives a very low ratio, whereas a mineral excipient, such as calcium phosphate (Aureomycin), gives a much larger ratio. The presence of mineral ash nearly always results in increased radioactivity. This is well shown in the case of the two antibiotics, Aureomycin and chloramphenicol, exposed in pure crystalline forms as well as capsular form. Column 2 shows that the crystalline forms have a very low ash content as compared with the capsular. Referring to column 4, which shows the respective microcuries of activity on a daily intake basis, it can be seen that Aureomycin in capsular form supplies nearly 100 times the radioactivity of the crystalline, while chloramphenicol supplies approximately 30 times.

With the exception of Aureomycin, which is included in this table for comparison purposes, none of the remaining drugs exhibited any activity in excess of the tolerance for P^{32} or S^{35}. Calculations also showed that Na^{24}, K^{42}, and Cu^{64}, all with half-lives ranging between twelve and fifteen hours, could not have contributed any measurable activity at 30 days.

As a matter of interest, readings on the ash samples were continued for four months. Radio-

TABLE IV.—DECAY IN INDUCED RADIOACTIVITY

	Radioactivity — Milliroentgens/Hr[a] After — 2 Days	5 Days	Apparent Half-life	Significant elements — Na	P	S	Br
Methantheline bromide	17	0.5	1.0	+	0	+	+
Phenobarbital	3.0	1.2	3.8	0	+	0	0
Thiopental sodium	5.5	0.5	1.5	+	0	+	0
Prantal methyl sulfate	0.9	0.8	29	0	+	+	0
Sulfadiazine	0.9	0.5	5.8	0	+	0	0
Aureomycin	5.0	2.0	3.8	0	+	+	0

[a] Beta-gamma survey meter, tube shield open.

Fig. 1.—Darkening of glass from exposure to nuclear explosion. A glass container from a heavily exposed box is compared with a control.

activity persisted, although at levels that could hardly be regarded as significant. Spectroscopic and chemical analyses, as well as the known formulations of the drugs, revealed tin, zinc, calcium, and iron. Theoretically, by reason of the longer half-lives of their radioactive isotopes, these species could be expected to contribute to the residual activity. Later information, however, has shown that under the conditions of these tests, calcium and iron do not become significantly radioactive.

An interesting example where tin presumably contributes to residual radioactivity is in tygon tubing. This was part of a transfusion kit exposed with each dextran bottle. Ash from this tubing contained radioactivity of 2×10^{-3} microcuries per gram at 30 days, with an estimated half-life of the order of 100 days. Spectroscopic analysis revealed the presence of tin from which a radioactive isotope, Sn^{113}, with a half-life of 105 days can be produced. While tin occurs in more than traces in tygon tubing (it is added in the curing process), trace quantities of this element were found in many of the drugs, presumably due to the fact that some pharmaceutical equipment is tin-lined.

In seeking to explain the radioactivity of the isotonic saline, at D + 30 neither the short-lived sodium 24 (0.62 D), nor the long-lived Cl^{36} (2×10^6 years) can account for the observed half-life of 109 days. However, the transmutation of the stable Cl^{35} isotope to the radioactive S^{35} isotope by an n,p reaction is a distinct possibility. *It may be assumed, therefore, that all of the drugs containing chlorine will be radioactive by virtue of S^{35} with a half-life of 87 days.* It should be emphasized that the other perfusion fluids, such as dextran, P. V. P., and the dextrose-salines, all have activities of the same order; they all contain chlorine and the calculated half-lives are within a range that is attributable to S^{35}.

In the light of the observations on the possible hazards of ingesting drugs within a week after exposure, it is apparent from Table VI that the 30-day decay period reduces the hazard even more. There is no question but that the ingestion of radioactivity is undesirable in general and should be avoided, particularly if exposure is to extend over many years. However, in cases of major disaster, such as envisaged here, the risk involved in a short-time use of drugs having no more activity than levels found in these experiments is quite negligible. In addition, the tolerance level for radioactivity is much higher under emergency conditions and for a short time than for long-continued exposure.

Chemical Tests.—Drugs from a box exposed at the "critical distance" from ground zero were examined on a recording infrared spectrophotometer and the tracings compared against those developed from the "transit control drugs." It will be recalled that the transit controls consisted of three drug boxes which were shipped to Mercury, Nev., and returned to Washington without having been exposed to ionizing radiation. The following drugs were studied: meperidine hydrochloride, morphine

TABLE V.—SIGNIFICANCE OF THE INDUCED RADIOACTIVITY OF DRUGS

	Days After Exposure	Ingested Microcuries per Day[a]	Tolerance of Microcuries Radioactivity per day[b] Br^{82} 1×10^{-7}	P^{32} 2×10^{-5}	Na^{24} 8×10^{-4}	S^{35} 5×10^{-3}
Methantheline bromide	2	2.2×10^{-2}	S	...	NS	NS
	5	2.2×10^{-2}	S	...	NS	NS
	30	1.4×10^{-4c}	NS	...	NS	NS
Phenobarbital	2	2.0×10^{-2}	...	S	...	NS
	5	1.8×10^{-2}	...	S	...	NS
	30	6.8×10^{-3}	...	S	...	NS
Thiopental Na	2	1.7×10^{-1}	...	...	NS	NS
	5	4.6×10^{-2}	...	...	NS	NS
	30	3.2×10^{-4c}	...	...	NS	NS
Prantal $Me.SO_4$	2	1.9×10^{-2}	...	S	...	NS
	5	2.8×10^{-2}	...	S	...	NS
	30	1.1×10^{-3}	...	S	...	NS
Sulfadiazine	2	6.3×10^{-2}	...	S	...	NS
	5	2.2×10^{-2}	...	S	...	NS
	30	1.2×10^{-2}	...	S	...	NS
Aureomycin	2	2.5×10^{-1}	...	S	...	NS
	5	2.0×10^{-1}	...	S	...	NS
	30	9.1×10^{-2}	...	S	...	NS

[a] Based on the best estimate of an average daily dose of the drug.
[b] Based on the most recent maximum permissible average concentration of radioactive materials per milliliter of water (Federal Register, in press). For the purposes of this tabulation, daily consumption of 1 liter of water is assumed.
[c] Activity noted at 30 days is probably due to the presence of S^{35} with T 1/2 of 87 days.

TABLE VI.—INDUCED RADIOACTIVITY OF DRUGS THIRTY DAYS AFTER EXPOSURE

Drug Preparation	Ash[a] Ratio	Microcuries per ml. or Gm. Drug Prepn.	Microcuries Intake in 24 Hours
Vitamin D	5.7×10^{-2}	4.1×10^{-5}	2.7×10^{-5}
Vitamin B_{12}	...	6.3×10^{-5}	1.9×10^{-4}
Vitamin K	...	1.5×10^{-4}	7.5×10^{-5}
Folic acid	3.1×10^{-2}	5.7×10^{-5}	9.3×10^{-6}
Neperidine hydrochloride	5.5×10^{-2}	5.1×10^{-5}	2.7×10^{-4}
Morphine sulfate	6.9×10^{-4}	2.5×10^{-5}	1.0×10^{-6}
Pantopon	3.4×10^{-2}	...	...
Methadone hydrochloride	...	1.1×10^{-5}	5.3×10^{-5}
Aspirin	2.4×10^{-4}	9.3×10^{-6}	2.6×10^{-5}
Atropine sulfate	5.8×10^{-4}	4.1×10^{-5}	2.0×10^{-4}
Digifolin	...	3.5×10^{-6}	6.7×10^{-6}
Epinephrine hydrochloride	...	8.7×10^{-6}	8.7×10^{-6}
Insulin	...	3.7×10^{-7}	...
Protamine zinc insulin	...	6.2×10^{-5}	3.1×10^{-6}
Aureomycin capsule	4.9×10^{-1}	3.8×10^{-2}	9.1×10^{-2}
Aureomycin crystals	2.0×10^{-4}	1.4×10^{-3}	1.3×10^{-3}
Chloramphenicol capsule	3.2×10^{-4}	1.3×10^{-3}	1.6×10^{-3}
Chloramphenicol crystals	1.2×10^{-4}	5.1×10^{-5}	5.1×10^{-5}
Terramycin	1.0×10^{-4}	7.9×10^{-6}	7.9×10^{-4}
Penicillin, potassium G	2.0×10^{-1}	8.1×10^{-4}	1.4×10^{-4}
Penicillin procaine	...	4.7×10^{-6}	4.7×10^{-6}
Penicillin dibenzyl[b]	...	1.3×10^{-5}	1.4×10^{-2}
Bacitracin	2.1×10^{-2}	3.1×10^{-4}	3.8×10^{-4}
Streptomycin	4.7×10^{-3}	4.4×10^{-5}	4.4×10^{-5}
Isotonic sodium chloride	...	5.4×10^{-5}	1.1×10^{-1}
Dextrose 10% in water	...	4.5×10^{-7}	2.3×10^{-4}
Dextran	...	7.8×10^{-6}	3.9×10^{-3}
Polyvinylpyrrolidone (PVP)	...	3.3×10^{-6}	3.3×10^{-3}
Benzalkonium chloride	...	2.6×10^{-4}	...
Thioglycollate medium U. S. P.	...	2.8×10^{-5}	...
Dextrose-saline tygon contact	...	2.5×10^{-5}	5.0×10^{-3}
Dextrose-saline latex contact	...	2.8×10^{-5}	5.6×10^{-3}
Dextrose-saline nat. rubber stopper contact	...	3.0×10^{-5}	6.0×10^{-3}

[a] Ash ratio = milligrams of ash per milligram of drug preparation.
[b] Dibenzylethylenediamine dipenicillin G.

sulfate, Pantopon,® methadone hydrochloride, amyl nitrite, nitroglycerine, methantheline bromide, phenobarbital, thiopental sodium, Prantal methyl sulfate, aspirin, sulfadiazine, cortisone acetate, ethyl ether, crystalline Aureomycin, crystalline chloramphenicol, potassium penicillin G, and 10% dextrose solution. The overall sensitivity of these tests was such that a 10% deviation from normal could be detected. In no instance could any abnormality be detected, and it may be concluded, therefore, that gamma and neutron fluxes of high intensity produced no measurable change in chemical constitution detectable by this method. In view of the negative results from a heavy exposure, drugs from less exposed boxes were not examined.

TABLE VII.—EFFECT OF RADIATION FLUX ON THE POTENCY OF VITAMIN B_{12}

Radiation Flux Neutrons/cm.^{2} Sulfur	Radiation Flux Neutrons/cm.^{2} Gold	Gamma Roentgens	Concentration of Vitamin B_{12}, Micrograms/ml.
(Control)	(Control)	...	15.0
(Control)	(Control)	...	15.2
1.64×10^{9}	5.39×10^{10}	11,000	13.9
1.64×10^{10}	1.41×10^{12}	15,000	13.7
2.44×10^{9}	8.31×10^{10}	760	14.0
1.89×10^{11}	5.58×10^{12}	20,000	8.1
7.30×10^{10}	2.20×10^{12}	20,000	8.0
7.18×10^{10}	2.74×10^{12}	20,000	8.0

Biological Tests.—*Toxicity of Closure Materials.*—The question as to whether certain standard closure materials could, upon exposure to ionizing radiation, impart toxins or impurities to a standard perfusion medium consisting of dextrose-saline, was studied in the following way. The solutions of dextrose-saline that had been in contact with finely divided tygon plastic, gum rubber, and lacquered black rubber stoppers while being exposed, were injected into rabbits. This is a standard pyrogen test capable of detecting less than 0.05 micrograms of a pyrogen or physiological equivalent. Under these conditions the animals would also respond to toxins that might have been elaborated in the test

TABLE VIII.—EFFECT OF RADIATION FLUX ON THE POTENCY OF 40-UNIT INSULIN

Radiation Flux Neutrons/cm.^{2} Sulfur	Radiation Flux Neutrons/cm.^{2} Gold	Gamma Roentgens	Potency, %	Fiducial Limits $P = 0.95$
1.89×10^{11}	5.58×10^{12}	20,000	86.6	76–99
1.75×10^{10}	1.29×10^{12}	3,000	90.8	79–104
7.37×10^{10}	2.69×10^{12}	20,000	92.7	90–96

materials. *All tests proved to be negative.* Furthermore, these experiments demonstrated that the dextrose-saline contained no pyrogen of bacterial origin which might have developed had the standard commercial closures failed under impact of pressure changes. Other tests conducted with the dextrose-saline also proved uniformly negative. They were (*a*) blood-pressure response, dog; (*b*) electrocardiogram, dog; (*c*) guinea pig intestine.

Toxicity of Special Drugs.—Sulfadiazine, methantheline bromide, and morphine sulfate were tested by the guinea pig intestine method. *All tests were negative.*

Toxicity of Antibiotics.—(From three most heavily exposed boxes and a control box), crystalline Aureomycin, crystalline chloramphenicol, potassium penicillin G, and bacitracin were tested for toxicity by the standard mouse test used in the Division of Antibiotics in certification. *No toxicity was found within the limits of the test.*

Potency.—Antibiotics: (From three most heavily exposed boxes and a control box), Aureomycin capsules, Aureomycin crystals, chloramphenicol capsules, chloramphenicol crystals, potassium penicillin G, procaine penicillin (dibenzylethylenediamine dipenicillin G), and bacitracin, were assayed by the standard methods used by the Division of Antibiotics in its certification operation. *All antibiotics were up to labeled potency.* Epinephrine: (From three most heavily exposed boxes and a control box). According to the standard pharmacopeial test, *all samples were within the range of acceptable potency.* Limits of error ±8%. Digifolin®: (From three most heavily exposed boxes). According to the standard pharmacopeial test, *all samples were within the range of acceptable potency.* Fiducial limits of assay ±20%. $P = 0.95$. Vitamin D: (From a most heavily exposed box, an "intermediate" box, and a control box). According to the standard pharmacopeial test *all samples were within the range of acceptable potency.* Fiducial limits of assay ±15%. $P = 0.95$. Folic Acid: (From three most heavily exposed boxes, an "intermediate" box, and two control boxes). By an alternate chemical test for this vitamin, *all samples were within the range of acceptable potency.* Fiducial limits of assay ±5%. $P = 0.95$. Vitamin B_{12}: Visual observation showed that heavily exposed samples had changed color. Microbiological tests showed that the potency was also considerably reduced. The examination of a number of samples exposed at various distances showed that there is a good inverse relationship between potency and the amount of radiation flux. The results are shown in Table VII. It can be seen that under the highest flux, reduction in potency amounted to 50%. Independent tests under laboratory conditions conducted with irradiation by 150,000 r confirmed this finding. For the present, therefore, it may be assumed that vitamin B_{12} is sensitive to both neutron and gamma radiation. Insulin: Examination of heavily exposed insulin solution showed it to be darker than the standards. Bioassay by means of the standard rabbit blood-sugar test showed that the potency was also reduced by about 10 per cent. Here, also, a good inverse relationship between potency and the amount of radiation flux was shown to exist. The results are shown in Table VIII. Protamine Zinc Insulin: Bioassay by means of the standard rabbit blood-sugar test *showed no reduction in potency of heavily exposed samples.*

Bacteriological Tests.—Eighteen bottles containing the U. S. P. thioglycollate medium were incubated at 35° for two weeks. All were sterile, thus showing that the closures had resisted pressure changes during exposure and remained bacteriologically intact. Portions from heavily exposed bottles and from control bottles were inoculated with viable microorganisms. Normal growth was obtained in all cases within twenty-four hours. It was concluded that exposure to ionizing radiation did not change the nutrient properties of the medium.

Two samples of benzalkonium chloride were tested for their bacteriostatic potency. No difference was found between the exposed sample and the control.

SUMMARY

1. Selected drugs were exposed to nuclear explosions so as to be subjected to high levels of ionizing radiation and shock. The results are as follows: (*a*) Insulin was reduced in potency by about 10 per cent. (*b*) Vitamin B_{12} was reduced in potency by about 50 per cent. (*c*) No deterioration in any of the other drug preparations was observed.

2. Glass containers exhibited marked induced radioactivity which decayed rapidly. Heavily exposed clear glass showed a marked "darkening."

3. Many drugs exhibited induced radioactivity which was measurable several months after exposure.

4. A significant amount of the induced radioactivity in the drugs was traced to the presence of certain mineral excipients such as calcium phosphate.

5. There is evidence that all chlorine-containing drugs, such as the salines and some perfusion preparations, become radioactive by transmutation of some of the chlorine to sulfur 35.

6. Little or no radiological health hazard is involved in the use of the exposed drugs, under disaster conditions. Since, however, under normal conditions, long term exposure to radioactivity is undesirable, special precautions should be taken in the use of drugs high in sodium chloride and phosphorus content.

The Colorimetric Determination of the Individual Components of Acetylsalicylic Acid, Acetophenetidin and Caffeine Mixtures*

By R. E. PANKRATZ and F. J. BANDELIN

The determination of the individual components of mixtures of acetylsalicylic acid, acetophenetidin, and caffeine through colorimetric procedures is given. A single separation, that of acetylsalicylic acid, is required prior to its determination as the iron-salicylate complex. Phenacetin and caffeine are determined on the whole mixture without separation or extraction. The former is determined, after hydrolysis, by coupling of the diazotized *p*-phenetidin to produce a dye with β-naphthol while the latter is determined as the phosphomolybdate in acetone solution. The hydrolysis of acetylsalicylic acid and acetophenetidin may be carried on simultaneously and the colors for all three components can be rapidly developed and determined. The method is reproducible and simple and the results are well within the usual limitations of colorimetric methods. Tables are given showing recovery of the components from weighed mixtures and production lot tablets.

THE DIFFICULTY of devising an adequate method for the separation and assay of the individual components of aspirin, phenacetin, and caffeine combinations is reflected in the numerous publications of methods and in the fact that until very recently this combination has not appeared in any official compendium despite the widespread use and established therapeutic rationale of such mixtures.

Although numerous methods have been devised for the separation and assay of these individual components, none has been eminently satisfactory. Separation by extraction with immiscible solvents under various conditions has been the usual approach. Such a method, utilizing gravimetric methods of determination of the components after separation, appears in the British Pharmaceutical Codex (1). In this country the A. O. A. C. (2) method and the widely used method of Holt (3) are also dependent on the usual "shake out and weigh" procedures. Mutual contamination of the components after separation influences weighings in these methods with resulting inaccuracies while the larger samples required for bromometric (4) and iodometric (5) and the lack of specificity of these methods leave much to be desired.

In a recent monograph adopted by the National Formulary for capsules of acetylsalicylic acid, acetophenetidin, and caffeine, the assay consists of the bromometric determination of aspirin and the iodometric determination of caffeine after separation by extraction with immiscible solvents. The assay suffers the disadvantage of determining the acetophenetidin by difference rather than by chemical assay.

* Received November 23, 1955, from the Control Laboratory of Flint, Eaton and Company, Decatur, Ill.

Spectrometric schemes have been proposed for the determination of the components in the infrared (6, 7) and ultraviolet (8, 9) regions of the spectrum by determining the absorbances at two or more critical wavelengths and calculating the amounts of the components present by applying simultaneous equations. With these procedures, slight errors in readings at any one point makes for considerable errors in the final values. Although the infrared method requires no separation, the ultraviolet method requires separation of the aspirin from the acetophenetidin and caffeine.

An interesting method of separation recently developed by Higuchi and Patel (10) and by Banes (11) utilizes differential solvent elution of the components from a charged silicic acid or Celite column upon which they are adsorbed. The quantities used are small and elution from the column is quite critical. Because of the minute quantities involved, determination of the components is limited to ultraviolet absorption.

It is the purpose of this paper to outline the colorimetric determination of the three components with a minimum of separation. This is accomplished by the use of a modification of Daoust's (12) phosphomolybdic acid precipitation method for caffeine, the colorimetric determination of phenacetin by diazotization and coupling of *p*-phenetidin following hydrolysis (13). and the aspirin by the iron-salicylate color after extraction and hydrolysis (14).

EXPERIMENTAL

Reagents.—Phosphomolybdic acid, 10% aqueous solution; sulfuric acid, 10% solution; dilute hydrochloric acid, concentrated reagent diluted 1 to 10; ethanol, U. S. P.; acetone, reagent grade; ferric

nitrate reagent, 1 Gm. $Fe(NO_3)_3.9H_2O$ dissolved in 100 ml. of 1:100 nitric acid; potassium hydroxide, 5% solution in ethanol, freshly prepared; sodium nitrite, 1% aqueous solution; ammonium sulfamate, 5% aqueous solution; betanaphthol, 1% solution in acetone; sodium hydroxide-sodium carbonate buffer, 5 Gm. of sodium bicarbonate and 1 Gm. of sodium hydroxide in 100 ml. of water.

Standards.—Salicylic acid standard solution: an aqueous solution containing 2 mg. of salicylic acid per ml.; *p*-phenetidin hydrochloride standard solution: an aqueous solution containing 0.1 mg. of resublimed *p*-phenetidin hydrochloride per ml.; caffeine standard solution: an aqueous solution containing 1 mg. of caffeine per ml.

PROCEDURE

Acetylsalicylic Acid Determination.—A standard curve for salicylic acid is prepared by using 2 to 10 ml. of the standard salicylic acid solution, at 2 ml. increments, pipetted into each of five 100-ml. volumetric flasks, 5 ml. of ferric nitrate reagent is added, the flasks are shaken and diluted to 100 ml. with water. Absorbance is read at 525 mμ and plotted on the abscissa against concentration in μg./ml. on the ordinate.

For the determination of acetylsalicylic acid in tablets, accurately weigh a sample containing approximately 1 Gm. of acetylsalicylic acid into a 250-ml. separatory funnel containing 25 ml. of 5% sodium bicarbonate solution. Extract by shaking with three 25-ml. portions of chloroform, washing each portion of chloroform with another 15-ml. portion of 5% sodium bicarbonate solution and with 25 ml. of water. Discard the chloroform. Quantitatively transfer all the aqueous extracts to a 250-ml. volumetric flask, add 50 ml. of alcoholic potassium hydroxide, and warm on a steam bath for two hours. Allow to cool, add 5 ml. of concentrated hydrochloric acid, and dilute to volume with water. Dilute 20 ml. of this solution to exactly 100 ml. Pipet 5 ml. of this s tion into a 100-ml. volumetric flask, add 5 ml. of ferric nitrate reagent, dilute to volume with water, and determine the absorbance at 525 mμ. The amount of salicylic acid present in the colored solution may be read directly from the standard curve. The concentration of salicylic acid multiplied by 1.3043 gives the amount of acetylsalicylic acid.

Phenacetin Determination.—Prepare a standard curve by diluting 25 ml. of standard *p*-phenetidin hydrochloride solution to exactly 250 ml. with water. Aliquots of 5-ml. increments ranging from 5 to 25 ml. are pipetted into each of five 100-ml. volumetric flasks. To each flask add 1 ml. of dilute hydrochloric acid and 1 ml. of 1% sodium nitrite solution. Allow to stand for three minutes. Next add 1 ml. of 5% ammonium sulfamate solution and allow to stand for one minute. Add 20 ml. of sodium hydroxide-sodium bicarbonate buffer followed by 1 ml. of betanaphthol reagent. Mix well and allow to stand for fifteen minutes, add 40 ml. of acetone and mix. Dilute to volume with water and determine absorbance at 470 mμ. Plot the absorbance as the abscissa and the concentration as the ordinate to establish the standard curve.

To determine phenacetin in the mixture weigh accurately a powder sample containing approximately 0.1 Gm. of phenacetin and transfer to a 100-ml. volumetric flask. Add 20 ml. of 10% sulfuric acid and heat on a steam bath for three hours. Cool to room temperature and dilute to volume with water. Dilute 10 ml. to exactly 200 ml. with water. Transfer 10 ml. of the diluted solution to a 100-ml. volumetric flask, add 15 ml. of water, then 1 ml. of dilute hydrochloric acid followed by 1 ml. of 1% sodium nitrite solution. Mix and allow to stand for three minutes. Add 20 ml. of the buffer solution and proceed as directed above for the standard curve. The concentration of phenacetin is found by multiplying the concentration of *p*-phenetidin hydrochloride found by 1.033.

Caffeine Determination.—The caffeine standard curve is prepared by pipetting 2, 4, 6, 8, and 10-ml. aliquots of caffeine standard solution into each of five 25-ml. glass-stoppered centrifuge tubes and adjusting the volume of all tubes to 25 ml. Add 1 ml. of diluted hydrochloric acid followed by 1 ml. of phosphomolybdic acid solution. Mix and heat in a boiling water bath for fifteen minutes. Remove from the water bath, cool, and chill for twenty minutes in an ice bath. Centrifuge the precipitate and carefully decant the supernatant liquid. Disperse the precipitate in 3 ml. of ice cold diluted hydrochloric acid and centrifuge again. Decant and discard the acid. Dissolve the precipitate in 25 ml. of acetone and determine the absorbance in a spectrophotometer at 440 mμ. Absorbance is plotted against concentration to give a straight line.

To determine caffeine in mixtures with acetylsalicylic acid and acetophenetidin, an accurately weighed sample containing approximately 100 mg. of caffeine is placed into a 100-ml. volumetric flask. Add 70 ml. of water and shake vigorously for ten minutes to dissolve all the caffeine, dilute to volume and filter through fast paper. Pipet 5 ml. of this solution into a 25-ml. glass-stoppered centrifuge tube, dilute to 25 ml. with water and proceed with the determination as given above. From the absorbance of the color developed the amount of caffeine present may be read directly from the standard curve previously prepared.

DISCUSSION

Acetylsalicylic acid must be separated by the procedure given because the phenacetin and caffeine yield, under the conditions of hydrolysis for acetylsalicylic acid, products which also develop color with the ferric nitrate reagent. In the usually encountered mixtures of acetylsalicylic acid, phenacetin, and caffeine, the interference amounts to about 10% of the total color. Color reactions for the phenacetin are carried out on the whole mixture since there is no interference in these reactions.

The use of glass-stoppered centrifuge tubes in the determination of caffeine was found to prevent "creeping" of the precipitate. A single washing of the phosphomolybdic acid precipitate was found to be adequate.

The following specific absorbances E 1%/1 cm. were determined for the colored reaction products of the three components using a Beckman DU spectrophotometer: acetylsalicylic acid, 81.4; acetophenetidin, 820; caffeine, 10.3. Results of a

series of determinations conducted on an accurately weighed mixture of the three components in a ratio of acetylsalicylic acid 3.5, acetophenetidin 2.5, and caffeine 0.5 are given in Table I. Results of another series of determinations on compressed tablets of a production lot having the same ratio of the components is shown in Table II.

Colorimetric analysis avoids the carry-over and mutual contamination of the components which occurs with immiscible solvent separation nor are the methods affected by complex formation of the compounds with each other.

TABLE I.—RESULTS OF A SERIES OF DETERMINATIONS ON AN ACCURATELY WEIGHED MIXTURE OF COMPONENTS

3.5 parts acetylsalicylic acid, 2.5 parts acetophenetidin, 0.5 part caffeine.

Recovery, %		
Acetylsalicylic Acid	Acetophenetidin	Caffeine
100.0	98.8	100.8
99.1	99.2	100.0
99.4	100.8	98.6
100.3	100.0	100.8
99.4	100.0	100.0
100.0	99.2	98.6
100.3	100.4	98.6
99.4	99.6	100.0
100.3	100.0	100.8
100.3	100.8	98.6
Mean 99.8	Mean 99.8	Mean 99.7

TABLE II.—RESULTS OF A SERIES OF DETERMINATIONS ON COMPRESSED TABLETS OF ACETYLSALICYLIC ACID, ACETOPHENETIDIN AND CAFFEINE

Theory Found, %		
Acetylsalicylic Acid	Acetophenetidin	Caffeine
100.5	99.5	100.4
101.0	99.0	100.0
101.0	100.0	100.4
99.2	99.5	100.4
101.0	99.0	100.0
102.0	103.0	100.4
99.2	98.0	99.6
101.0	100.0	100.4
100.0	98.0	100.4
101.0	98.5	100.0
Mean 100.6	Mean 99.2	Mean 100.2

SUMMARY

1. The colorimetric determination of the individual components in mixtures of acetylsalicylic acid, acetophenetidin, and caffeine is described.

2. The single separation necessary is that of acetylsalicylic acid which is obtained in the aqueous phase after partitioning the components between chloroform and sodium bicarbonate solution.

3. The acetylsalicylic acid in this solution is determined as salicylate, after alkaline hydrolysis, by the iron-salicylate color.

4. Acetophenetidin is determined after hydrolysis to *p*-phenetidin by diazotization of the primary aromatic amino group and coupling with betanaphthol in alkaline solution to yield a red dye which is read in a colorimeter at 470 mμ.

5. The caffeine is determined by precipitation as the phosphomolybdate, dissolving the washed precipitate in acetone and determining the absorbance of the resulting solution in a colorimeter at 440 mμ.

6. The acetophenetidin and caffeine may be run in the whole mixture without separation since the color development is specific for each of these compounds in the mixture.

7. Specific absorbance for the colored derivatives of the three compounds are given and a series of determinations on the recovery of the components from an accurately weighed mixture and from production lot tablets are presented.

8. The method is applicable to both tablet and capsule formulations and medicaments commonly added to this mixture such as codeine, barbiturates, and antihistaminics do not interfere in any of the determinations.

9. Colorimetric analysis avoids carry-over and mutual contamination of components which occurs with immiscible solvent extraction and is not influenced by complex formation between compounds.

10. The methods have an accuracy well within the usual inherent variation of colorimetric methods and are reliable, reproducible, and require less time than the usual extraction procedures.

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Estrogenic Activity of Some Flavonoids*

By DUANE G. WENZEL† and PHILIP ROSENBERG‡

Six flavonoids were tested both orally and subcutaneously for estrogenic activity by the uterine weight increase method in the immature rat. One of the compounds, 4′,6-dihydroxyflavone, was active orally but not subcutaneously. Postulates are advanced to explain the results.

THE ISOFLAVONE PIGMENT genistein and its glycoside genistin both possess estrogenic activity (1, 2). Genistein, which is found in soybean meal (3) and subterranean clover (4), is 4.44×10^{-6} as potent in estrogenic activity as diethylstilbestrol (2). The 4′-methyl ether of genistein (5) and various synthetic relatives of genistein, both isoflavones and isoflavens, were also found to be active (6, 7, 8). Estrogenic substances have been reported in other plant materials as reviewed by Costello and Lynn (9).

Although the structural requisites for the estrogenic activity of a compound are relatively nonspecific, there are certain similarities in the configuration of the synthetic and natural estrogens and of the isoflavonoids. Probably the most obvious of these is the presence of hydroxyl or carbonyl groups at diametrically opposed ends of the molecule. With the possible exception of stilbene (10) all estrogenically active compounds have hydroxyls in opposition to one another or to a carbonyl or carboxyl group.

The work which follows was initiated in order to test the hypothesis that the isoflavone structure is not essential for estrogenic activity, but that other flavonoids having diametrically opposed hydroxyl groups either initially or through chalconoid formation may be estrogens.

EXPERIMENTAL

Materials.—Liqueritin was supplied by C. K. Swift of MacAndrews and Forbes Company. Liqueritigenin was prepared from the glycoside according to Shinoda and Ueeda (11). The liqueritigenin melted at 204–207°, reported 207° (11). Osajin and pomiferin were extracted and purified from the dried meal of the Osage orange by the methods of Walter, *et al.* (12), and Wolfram, *et al.* (13), respectively. The osajin melted at 189–191°, reported 189° (12); pomiferin melted at 199–201°, reported 200.5° (13). Naringenin and hesperetin were obtained from C. W. Wilson, of Sunkist Growers. The 4′,6-dihydroxyflavone was synthesized by the procedure of Konstanecki, *et al.* (14, 15). The compound melted at 318–321° while that reported was 320° (15).

Method.—Immature female rats were used to test for estrogenic activity according to a procedure modified from that of Lauson, *et al.* (16). Fourteen experimental groups of 8 female Sprague Dawley rats from twenty-one to twenty-three days old were used. All animals were obtained from litters having at least four females each and members of the same litters were randomly distributed so that littermates were in separate groups. Estrone was administered subcutaneously in 2-μg. doses while the flavonoids were given in doses of 2.5 mg. subcutaneously and 5 mg. orally. Each dose for subcutaneous administration was contained in 0.2 cc. of cottonseed oil while the oral preparations were contained in 0.2 cc. of a 0.1% aqueous tragacanth suspension. Drugs were administered at 8:00 a. m. and 4:00 p. m. daily for four days. The total dosage administered per animal was 0.016 mg. of estrone, 20 mg. of the flavonoids, subcutaneously, and 40 mg. orally. A control group was injected with 0.2 cc. of cottonseed oil on the same schedule. On the fifth day, ninety-six hours after the first injection, the rats were sacrificed, their uteri removed, weighed, and measured. The data obtained were analyzed for statistical significance by the difference of means method (17).

RESULTS AND DISCUSSION

From the values in Table I, it is seen that there were only two experimental groups with mean uterine weights that were significantly larger than those of the control. These were the estrone and the subcutaneous 4′,6-dihydroxyflavone groups. There was no significant change in any of the mean uterine lengths. It is clear that the isoflavone structure *per se*, as found in the active genistein and the inactive osajin and pomiferin, is not the sole basis for estrogenic activity of the flavonoids. However, before attempting to come to any conclusions with regard to structure and estrogenic activity of the flavonoids it would be well to summarize the findings of others in this regard.

Bradbury and White (6) and Baker, *et al.* (8), found that the estrogenic activity of genistein was either diminished or lost upon the introduction of alkyl groups in the 2-position or blocking of the 4′- or 7-position with methoxyl groups. Furthermore, all the isoflavones they found to be active had a hydroxyl group in the 5-position. It has been suggested that the 2-alkyl substitution interferes with the coplanarity of the genistein molecule while the presence of the 5-hydroxyl group decreases the double bond character of the chromone carbonyl group (6). These same workers (6) found that

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when the 2,3-isoflavone double bond of genistein was reduced to form the corresponding isoflavanone, activity was lost. When the 2,3 double bond was in the 3,4-isoflaven position, however, the structure-activity relationship markedly changed (6, 7). The parent compound, 4′,7-dimethoxyisoflaven, of the series that Bradbury and White (6) and Bradbury (7) tested was inactive, but its 2- or 4-alkyl or aryl derivatives were all estrogens. This may be explained by the structural similarity of the 2 or 4 substituted isoflavens to diethylstilbestrol as shown in the following formulas.

Of the structure-activity relationships that may be deduced from the present study, probably the most significant is the fact that while the estrogenically active 4′,6-dihydroxyflavone is not an isoflavone it does resemble genistein in possessing diametrically opposed hydroxyl groups. A second point of interest is that in previous work (1) others found that genistein was active both orally and upon subcutaneous administration. However, 4′,6-dihydroxyflavone is active only upon subcutaneous injection. A possible explanation for this difference may be that 4′,6-dihydroxyflavone is not absorbed from the gastrointestinal tract. It seems more likely that in accord with the apparent need for opposing hydroxyl groups, the results may be explained on the basis of chalconoid formation. Haley (18) has

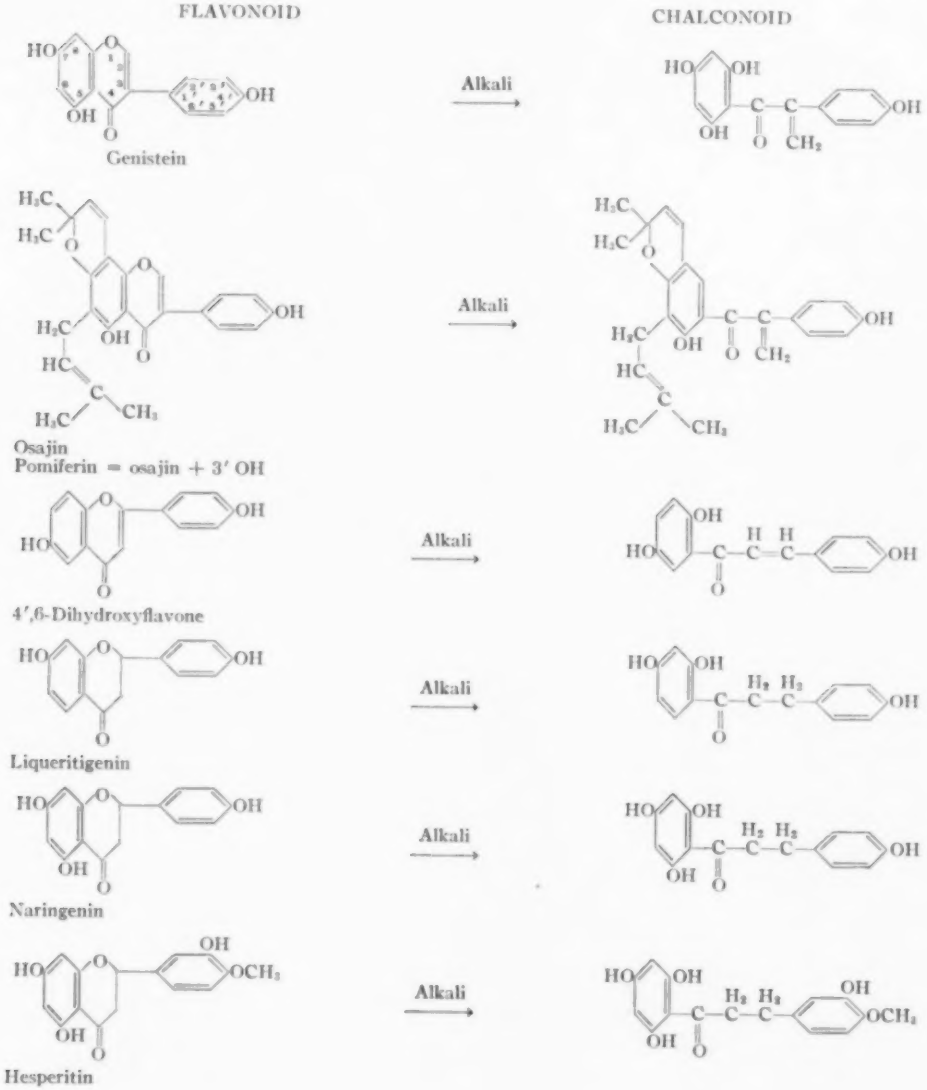

TABLE I.—THE EFFECT OF FLAVONOIDS ON THE IMMATURE RAT UTERUS

Compound	Mean Uterine Weight in mg. ± S.E.	Mean Uterine Length in mm. ± S.E.
Control (cottonseed oil)[a]	36 ± 2.5	63 ± 6.8
Estrone[a]	133 ± 5.8[c]	72 ± 7.7
Osajin[a]	39 ± 2.1	63 ± 6.2
Osajin[b]	31 ± 3.9	61 ± 4.6
Pomiferin[a]	26 ± 1.6	55 ± 5.2
Pomiferin[b]	30 ± 3.3	60 ± 5.9
4′,6-Dihydroxyflavone[a]	62 ± 3.4[c]	69 ± 2.6
4′,6-Dihydroxyflavone[b]	37 ± 3.4	65 ± 1.3
Liquiritigenin[a]	42 ± 1.4	64 ± 1.5
Liquiritigenin[b]	35 ± 4.2	66 ± 2.5
Naringenin[a]	39 ± 1.7	69 ± 1.5
Naringenin[b]	37 ± 2.2	67 ± 4.4
Hesperitin[a]	40 ± 2.2	60 ± 1.9
Hesperitin[b]	33 ± 2.3	64 ± 1.9

[a] Subcutaneous, total dose 1.6 cc. cottonseed oil, 0.016 mg. estrone, 20 mg. flavonoid.
[b] Oral, total dose 40 mg. flavonoid.
[c] $P < 0.01$.

indicated that the "vitamin-P-like" activity of the flavonoids is based upon their conversion to chalconoids in the body. If, therefore, the flavonoids form chalconoids in the alkaline intestine, but not when injected, and if the relative position of the hydroxyl groups is critical, then genistein having the hydroxyl groups diametrically opposed in both the parent structure and the chalconoid would be active both orally and upon injection as has been reported (1). On the other hand, while the hydroxyl groups of 4′,6-dihydroxyflavone are theoretically in the correct positions in the unaltered structure, this is no longer true upon chalconoid formation and could account for the lack of oral activity.

If chalconoid formation does occur, then the carbon bridge length and/or configuration are also critical, as the chalconoids of liquiritigenin, naringenin, and hesperetin have diametrically opposed hydroxyl groups yet these flavonoids are inactive estrogenically.

From the experimental evidence it seems likely that both the position of the hydroxyl groups and the chain length and configuration of the chalconoid bridge are critical for estrogenic activity of the flavonoids.

SUMMARY

1. Six flavonoids were tested orally and subcutaneously for estrogenic activity in the immature female rat. The only active member of the group was subcutaneously administered 4′,6-dihydroxyflavone.

2. This compound is related to the estrogenically active isoflavonoids and the synthetic and natural estrogens in possessing hydroxyl groups on diametrically opposed ends of the molecule.

3. The lack of oral activity by 4′,6-dihydroxyflavone may be the result of chalconoid formation in the intestinal tract with loss of the opposed position of the hydroxyl groups. The chalconoids of the other flavonoids tested are postulated as being inactive either because of the lack of diametrically opposed hydroxyl groups or lack of proper carbon bridge length or configuration.

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The Effect of Some Selected Surface-Active Agents on the Extraction of Cape Aloe*

By NOURI Y. MARY,† BERNARD V. CHRISTENSEN,‡ and JACK L. BEAL§

A study of the effect of some surface-active agents on the extraction of Cape aloe was undertaken. Of the surface-active agents investigated, the results indicated that the nonionic and cationic surface-active agents increased the amount of free and combined anthracene derivatives extracted from Cape aloe. The anionic surface-active agent was not suitable for the purpose.

SEVERAL procedures for the extraction of the constituents of crude drugs appear in the literature. Among these are the Waring blendor (1), the extraction cooker (2), and the colloid mill (3). Most recently the introduction of some surface-active agents into the extracting menstruum indicated their value in the extraction of alkaloidal crude drugs (4, 5). A preliminary study conducted at these laboratories indicated the possibility of utilizing those agents in the extraction of a glycosidal drug. Cape aloe was used as the glycosidal drug. The following investigation was, therefore, undertaken to study in more detail the efficiency of nonionic, anionic, and cationic surface-active agents in the extraction of Cape aloe, when introduced into the extracting menstruum in small concentration.

EXPERIMENTAL

Materials.—Cape aloe,[1] Tween®80,[2] (a nonionic surface-active agent); Tergitol®,[3] (an anionic surface-active agent); and Cetab®,[4] (a cationic surface-active agent).

Procedure.—Aqueous extracts of Cape aloe were prepared according to the U. S. P. XIV method (6) using 0.25%, 0.50%, and 1.0% concentrations each of Tween 80, Tergitol 08, and Cetab. Control samples were prepared simultaneously for the purpose of comparison. The aqueous extracts were evaluated for the amount of water-soluble extractive, surface tension, and *p*H. The amount of water-soluble extractive was determined according to the U. S. P. XIV method (6). Surface tension measurements were carried out by means of a Cenco-Du Nouy tensiometer, precision direct reading model, Serial No. 509. A Beckman model G *p*H meter was used for the determination of the *p*H values. The results of all experiments appear in Tables I, II, and III.

As can be seen from these three tables, more water-soluble extractive was obtained by the treatment of Cape aloe with the various concentrations of the surfactants than was obtained from the control samples. The question then arose whether this extra extractive was of an active nature or whether it consisted merely of inert material. It was, therefore, thought that some guidance might be obtained on this problem, by estimating the amount of free and combined anthracene derivatives present in the various water-soluble extractives obtained.

The method used for the estimation of the free and combined anthracene derivatives was substantially that of Kussmaul and Becker (7), modified later by Forsdike (8). The results of the analyses are listed in Tables I, II, and III.

DISCUSSION

In reference to Table I, it can be seen that Tween 80 in 0.25%, 0.50%, and 1.0% concentrations removed more water-soluble extractive than the control. The extra water-soluble extractives were of an active nature. The results showed that there was a marked increase in the amount of free and combined anthracene derivatives extracted with any concentration of Tween 80 added. The increase in the amount of free and combined anthracenes was in direct proportion to the concentration of Tween 80 employed.

With Cetab, a cationic surfactant, results somewhat similar to the Tween 80 experiments were obtained. Examination of the data in Table III reveals that more water-soluble extractive was yielded with various concentrations of Cetab than with the control. Also, these extra water-soluble extractives contained more free and combined anthracene derivatives than the control samples. It is to be noticed, however, from a comparison of the data in Tables I and III that Cetab with each concentration used, extracted significantly less water-soluble extractive and almost as much free and combined anthracene derivatives as the corresponding concentration of Tween 80. Hence, the menstruum containing the cationic surface-active agent, Cetab, extracted less inert material than the menstruum containing the nonionic surface-active agent, Tween 80, and therefore the menstruum containing Cetab would be preferred. Unlike Tween 80, Cetab did not increase the amount of combined

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[1] Obtained from S. B. Penick & Co., 50 Church St., New York, N. Y.
[2] Courtesy of Atlas Powder Co., Wilmington, Del.
[3] Courtesy of Carbide and Carbon Chemicals Corp., 30 E. 42nd St., New York, N. Y.
[4] Courtesy of Rhodes Chemical Co., 417 Cleveland Ave., Plainfield, N. J.

TABLE I.—EXTRACTIONS WITH TWEEN 80[a]

Concn., %	Water-Soluble Extractive, Gm. %	Free Anthracene Derivatives, mg. %	Combined Anthracene Derivatives, mg. %	Surface Tension, dynes/cm.	*p*H
Controls	68.95	193	423	56.2	4.67
0.25	81.15	228	481	42.5	4.60
0.50	86.87	265	496	42.6	4.65
1.00	97.92	295	539	42.0	4.75

[a] Results are averages of 3 determinations.

TABLE II.—EXTRACTIONS WITH TERGITOL 08[a]

Concn., %	Water-Soluble Extractive, Gm. %	Free Anthracene Derivatives, mg. %	Combined Anthracene Derivatives, mg. %	Surface Tension, dynes/cm.	*p*H
Controls	70.04	193	423	54.9	4.67
0.25	77.59	351	371	47.8	5.65
0.50	81.70	413	354	44.4	5.95
1.00	84.12	441	385	44.2	6.40

[a] Results are averages of 3 determinations.

TABLE III.—EXTRACTIONS WITH CETAB[a]

Concn., %	Water-Soluble Extractive, Gm. %	Free Anthracene Derivatives, mg. %	Combined Anthracene Derivatives, mg. %	Surface Tension, dy nes/cm.	*p*H
Controls	69.29	193	423	55.6	4.67
0.25	73.64	215	497	43.8	4.00
0.50	81.71	243	472	43.1	3.95
1.00	89.39	262	555	40.9	3.92

[a] Results are averages of 3 determinations.

anthracenes in direct proportion to the concentration employed.

Some unusual results were obtained in using Tergitol 08, an anionic surface-active agent. Although it extracted consistently more water-soluble extractive with increasing concentrations than the control, the analysis of the free and combined anthracenes showed results different from the previous two surface-active agents. Table II reveals that there was a very marked increase in the amount of free anthracenes as compared with the control samples. Apparently, Tergitol 08 in some way hydrolyzed or broke the glycosidal combination of the anthracenes and thus increased the amount of free anthracenes at the expense of the combined ones. This breaking down of the glycosidal anthracene derivatives is not desirable since experimental data recorded in the literature (9) indicate that the purgative activity of the anthracene derivatives is greatest when they are in the glycosidal form. Tergitol 08, therefore, seems to be unsuitable for the extraction of Cape aloe.

The mechanism by which the efficiency of extraction is increased by the use of surfactants is not known. Surface tension and *p*H measurements did not give any satisfactory explanation of such a mechanism. The surface tension was decreased to the same extent with all concentrations of surfactants employed, yet the amount of free and combined anthracenes increased proportionally with the increase in concentration. The *p*H data as well did not offer any convincing interpretation. The increase in extraction efficiency is probably due to increased solubilization in the presence of the surface-active agents.

CONCLUSION

Of the surface-active agents investigated, the nonionic and cationic surface-active agents in an aqueous menstruum appear to be useful in increasing the extraction efficiency of the free and combined anthracene derivatives of Cape aloe. The anionic surfactant, however, is not recommended for this purpose. The *p*H and surface tension of the menstruums were not appreciably altered and could not be factors in explaining the efficiency of the extraction.

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The Effect of Triterpenes on the Excretion of Sodium and Potassium by Rats*

By DUANE G. WENZEL† and GILBERT Y. KOFF‡

The acetates of ursolic acid, oleanolic acid, glycyrrhetinic acid, lupeol, and lurenol were administered subcutaneously to adrenalectomized rats and their effect on the excretion of sodium and potassium determined. Ursolic acid acetate induced significant sodium retention at 3 mg. per rat. Potassium excretion was not affected.

SEVERAL TRITERPENES have been tested and found to be devoid of hormonal action as evaluated by the cold stress test, glycogen deposition, and uterine or prostate weight gain (1). Glycyrrhetinic acid was found to influence sodium and potassium excretion by Hassan, *et al.* (2) and Galal (3) although the latter indicated the effect to be antidiuretic in nature. Other workers (4, 5) found no evidence of an effect upon electrolyte excretion.

It was the purpose of this investigation to determine the effect of the triterpenes originally tested (1) on the excretion of sodium and potassium by rats.

EXPERIMENTAL

Materials.—Sources of the triterpenes tested: ursolic acid, oleanolic acid, glycyrrhetinic acid, lupeol, and lurenol, are described in a previous publication (1).

Method.—The procedure of Marcus, *et al.* (6), for the study of electrolyte excretion was adopted with modifications. Male Sprague-Dawley strain rats weighing between 130 and 170 Gm. were used. These were adrenalectomized under pentobarbital anesthesia, 30 mg./Kg., i. p. After adrenalectomy and for the first postoperative day animals were given Purina Laboratory Chow and physiological saline *ad libitum*. On the second postoperative day, forty-eight hours after adrenalectomy, tap water was substituted for the saline. Twenty-four hours later food and water were withdrawn for four hours. Immediately following this fasting period the acetates of desoxycorticosterone, ursolic acid, oleanolic acid, glycyrrhetinic acid, lupeol, and lurenol were administered subcutaneously. The dose of the triterpenes was 3 mg. and that of DCA was 3 μg. per animal. These injections were immediately followed by the intraperitoneal administration of 5 ml. of 0.9% sodium chloride solution. The bladders were emptied under etherization by suprapubic pressure and the urine discarded. The rats were then placed in pairs in commercial rat metabolism cages. Fine screening was placed beneath the flooring to catch the feces. Urine was collected in 100-ml. flasks for four hours. During the period of collection the room was kept in semidarkness and the rats were not disturbed. In order to further insure the consistency of the results, assay periods were begun at the same time each day to avoid diurnal variations. At the end of the four hours the bladders were again emptied and the urine added to that which had been collected. Because the total urine volumes were small, the errors due to evaporation were greatly magnified and the urine volumes were not used. Instead, the collecting surfaces which had previously been rinsed free of sodium and potassium, were washed with distilled water. The washings were added to the urine and the volume brought up to 100 ml.

The 100-ml. urine dilution of each pair of animals was analyzed for its sodium and potassium content by use of a Perkin-Elmer flame photometer, model 52-C. The results are given in Table I as the mean of the electrolyte excretions of the rat pairs. Values are given simply as p. p. m. of the electrolyte excreted over the four-hour test period in the 100-ml. dilution without regard for body weight as all groups were of approximately equal average weight. The potassium-sodium ratio is also given as it is believed that this figure is a more sensitive criterion of the influence of a drug on sodium and potassium excretion than are the ion concentrations alone (7).

RESULTS AND DISCUSSION

From the mean ion concentrations and the potassium-sodium ratios in Table I it is apparent that DCA and ursolic acid acetate have produced significant (8) effects on sodium and potassium excretion that were comparable at a thousand-fold difference in the dose levels. No attempts were made, however, to run quantitative determinations. The remaining triterpenes and the control group determinations do not differ significantly (8). Inspection of the figures reveals that the increased potassium-sodium ratios of DCA and ursolic acid acetate are almost entirely due to the sodium retention as potassium excretion does not appear to be appreciably affected.

In view of the fact that no alteration of sodium and potassium excretion could be demonstrated with glycyrrhetinic acid acetate and that others (2) have found such activity, it is advisable to consider possible reasons for this apparently anomalous result. An obvious point of difference is the method of administration. Hassan, *et al.* (2), gave glycyrrhetinic acid and other products of glycyrrhiza orally, while the present study involved only subcutaneous administration. A second factor may have been the size of the dose. In these experi-

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TABLE I.—SODIUM AND POTASSIUM EXCRETION IN ADRENALECTOMIZED RAT FOLLOWING SUBCUTANEOUS ADMINISTRATION OF TRITERPENES

Drug[a]	Pairs per Drug	Dose per Rat	Sodium p. p. m. ± S.E.	Potassium p. p. m. ± S.E.	Potassium-Sodium Ratio
Control (sesame oil)	5	...	240 ± 75	41 ± 3.4	0.171
Desoxycorticosterone	8	3 μg.	66 ± 28[b]	42 ± 7.6	0.636
Ursolic acid	4	3 mg.	70 ± 38[b]	54 ± 14.0	0.714
Oleanolic acid	6	3 mg.	240 ± 59	48 ± 14.1	0.200
Glycyrrhetinic acid	6	3 mg.	260 ± 64	42 ± 5.8	0.162
Lupeol	7	3 mg.	200 ± 46	41 ± 4.5	0.205
Lurenol	6	3 mg.	220 ± 40	46 ± 8.2	0.209

[a] As the acetates. [b] Significant at 1 to 5% level. S. E. = $\sqrt{\frac{\Sigma d^2}{N(N-1)}}$.

ments ursolic acid induced marked sodium retention at a dosage level of 3 mg. while the studies of the workers previously mentioned indicated a minimal, if any, action for glycyrrhetinic acid at doses of 200 mg. Inasmuch as they found other products of glycyrrhiza to have somewhat more activity than glycyrrhetinic acid it is also possible that these materials would have been active under the conditions of administration and dosage used in this study. The recent finding by Galal (3) that glycyrrhetinic acid affects sodium excretion by an antidiuretic effect would make it advisable to determine the effect of ursolic acid on diuresis.

The explanation for the difference in the activity of ursolic acid and the other triterpenes tested is not readily apparent from an examination of the structures. It may be conjectured that the activity of ursolic acid acetate is not the result of the compound *per se*, but of a biotransformation product. Ursolic acid under chemical treatment will form a structure having the α,β-unsaturated carbonyl group characteristic of so many steroidal hormones (9). Such a configuration is, of course, present in glycyrrhetinic acid. There is evidence to indicate that the body can dehydrogenate compounds with reduced rings and can introduce carbonyl groups (10).

The fact that ursolic acid inhibits sodium excretion is of practical interest because this compound is finding extensive use in the food and drug industries as an emulsifying agent. In the quantities used for such purposes it may present a health hazard, particularly in individuals for whom sodium is interdicted. Ursolic acid is relatively cheap and although it does not possess the stress-protecting action of DCA (1), it may find therapeutic application in conditions such as Addison's disease.

SUMMARY

1. The acetates of ursolic acid, oleanolic acid, glycyrrhetinic acid, lupeol, and lurenol were tested for their effect on the electrolyte excretion of adrenalectomized rats.

2. Ursolic acid acetate administered subcutaneously in a dose of 3 mg. induced sodium retention comparable to that of DCA in a dose of 3 μg. Potassium excretion was not significantly affected.

3. Possible explanations for the results and the medicinal implications are discussed.

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The Relation between *in Vitro* Disintegration Time of Sugar-Coated Tablets and Physiological Availability of Sodium *p*-Aminosalicylate*

By D. G. CHAPMAN, R. CRISAFIO, and J. A. CAMPBELL

A study was carried out to relate *in vitro* disintegration time of coated tablets with physiological availability of sodium *p*-aminosalicylate based on the urinary excretion of this drug by human subjects. It was found that tablets having *in vitro* disintegration times of longer than sixty-seven minutes were not completely available. Eight-hour urinary excretion of sodium *p*-aminosalicylate gave the same general indication of availability as did the twenty-four- or thirty-two-hour collections and was equally precise. The results support earlier work based on the urinary excretion of riboflavin. The combined data suggest that the maximum time permitted for the *in vitro* disintegration of coated tablets to ensure complete availability should be sixty minutes.

RIBOFLAVIN has been used satisfactorily as a marker in physiological availability studies (1, 2). Work in this laboratory confirmed the reliability of this bioassay technique (3), and showed that sugar-coated tablets which had *in vitro* disintegration times of longer than sixty minutes were not completely available to human subjects as judged by urinary excretion of riboflavin (4). However, since only approximately fifty per cent of a given dose of riboflavin was excreted in the urine, it was suggested (5) that some other drug might have given a somewhat different picture of the physiological availability of the contents of a tablet.

The report by Way, *et al.* (6), that *p*-aminosalicylic acid is largely and rapidly excreted, and could be readily determined in the urine suggested that this substance might serve as a useful marker for a physiological study. Accordingly, in order to accumulate more evidence of the relationship between *in vitro* disintegration time and physiological availability it was decided to use sodium *p*-aminosalicylate (Na PAS) as a marker. It is the purpose of this paper to present the results of such a study.

METHODS

Physiological Availability.—Both free and total Na PAS were determined in the urine using the method of Way, *et al.* (6). The amounts found in the urine were considered to reflect the availability of sodium *p*-aminosalicylate from a particular tablet or standard. All data reported in this paper are in terms of total Na PAS.

Subjects.—Seven normal male employees of the Food and Drug Laboratories ranging in age from twenty-five to forty-two years volunteered as subjects. Each subject continued to consume his regular meals.

Sodium *p*-Aminosalicylate Standards.—Two standards of Na PAS ($C_7H_6O_3NNa \cdot 2H_2O$) were used for the availability studies. One was a solution, prepared just before use, containing 500 mg. in 50 ml. of distilled water and the other a hard gelatin capsule containing 250 mg. having an *in vitro* disintegration time of 3 minutes. In either case, a total of 500 mg. of the drug was administered as the test dose.

Sodium *p*-Aminosalicylate Tablets.—Seven different sugar-coated tablets were used in this study. Three of these tablets, B, E, and G, were obtained from one company and contained 330 mg. of Na PAS. These three tablets were prepared to provide varying disintegration times. Tablets A, C, D, and F were market products from four other pharmaceutical firms and were reported to contain 500 mg. of Na PAS. The test subjects were given two of the 330-mg. or one of the 500-mg. tablets to determine physiological availability. All tablets were assayed for Na PAS prior to administration.

Assay Periods.—The Na PAS standard or tablet was ingested at 9 a. m. which was approximately one and one-half hours after breakfast. The urine was collected at 2-hour intervals for the first eight hours. The following sixteen-hour (overnight) and eight-hour (next day) periods were also collected, to give a total of thirty-two hours collection from the time that the material was ingested. In the sixteen- and eight-hour collection bottles 10 cc. of 1% nitric acid was added.

Disintegration Test.—The apparatus, media, and procedure used in carrying out the disintegration test has been described previously (4). In addition, all tablets were disintegrated using U. S. P. XV fluids (7) and clear plastic disks as described by Vliet (8). The clear plastic disks measured 21 mm. in diameter, 9.8 mm. in thickness and weighed 3.95 Gm. and moved through a distance of about 2 cm. Six tablets from each of the seven samples were dis-

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integrated on three different days by both procedures.

RESULTS

Physiological Availability.—The urinary excretion of Na PAS following the ingestion of the standards or tablets was determined for each of the seven individuals. The total amount of Na PAS appearing in the urine was considered to reflect the availability of this material from the standards or tablets. The mean rates at which the Na PAS was excreted in the urine are shown in Fig. 1. As the two standards were excreted at practically identical rates, only one curve, that represented by open circles, is used to illustrate both excretions. In the same manner, Na PAS was excreted from tablets B, C, and D in similar amounts and rates and hence the curve for tablet D has been used to represent all three tablets.

In Table I the percentage excretions of the individual subjects following the ingestion of the standards and tablets are shown. It can be seen that Na PAS is rapidly excreted following the ingestion of the standard solution, with the largest percentage, 45.6%, appearing in the urine in the first two-hour period. The amounts excreted continued to rise more slowly until at the eight-hour period 68.9% had been excreted and 72.5% in twenty-four hours. A further 1.1% was excreted between the twenty-four and thirty-two hour period to bring the total excretion to 73.6% following the ingestion of 500 mg. The excretion following the ingestion of the Na PAS capsule was very similar to the excretion obtained following the ingestion of the solution of Na PAS.

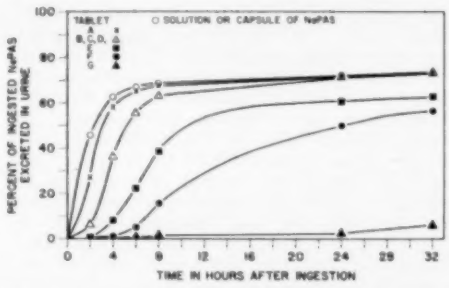

Fig. 1.—Relationship between time and per cent of dose of Na PAS excreted in the urine.

The excretion of total Na PAS following the ingestion of each of the seven tablets, varied over a wide range. The excretion of Na PAS from tablet A was

TABLE I.—PER CENT OF DOSE OF SODIUM PARA-AMINOSALICYLATE EXCRETED FOLLOWING THE INGESTION OF THE STANDARD OR TABLETS

Form of PAS →	Na PAS solution						Na PAS capsule						Tablet A					
Hours after ingestion →	2	4	6	8	24	32	2	4	6	8	24	32	2	4	6	8	24	32
Subjects:																		
1	43.2	62.4	65.2	67.8	76.0	77.4	34.2	57.4	60.6	64.6	72.6	76.4	33.4	58.8	63.0	68.6	75.4	75.4
2	53.4	68.2	71.6	73.2	75.0	75.0	37.6	59.4	66.2	68.6	72.4	73.4	1.2	48.6	60.4	67.2	71.4	71.8
3	61.2	75.0	79.0	80.2	81.4	83.0	50.8	63.8	69.0	70.8	76.2	78.8	38.8	68.8	73.4	75.2	79.2	81.0
4	35.2	55.4	63.4	65.8	69.2	70.2	37.2	52.2	65.4	68.6	73.0	74.4	26.2	60.4	66.0	68.6	72.6	74.0
5	47.2	62.2	65.2	66.8	70.0	71.2	51.4	67.0	70.6	72.6	77.8	80.0	38.8	62.2	66.4	68.2	72.0	73.4
6	46.8	62.6	68.6	70.2	73.8	73.8	51.0	68.4	71.2	73.4	76.0	76.0	5.4	49.0	64.8	67.4	72.2	73.4
7	32.2	52.2	56.4	58.6	62.4	64.4	26.6	53.0	57.4	59.8	64.4	66.0	46.2	60.2	62.2	62.2	64.0	65.6
Mean →	45.6	62.6	67.1	68.9	72.5	73.6	41.3	60.2	65.8	68.3	73.2	75.0	27.1	58.3	65.2	68.2	72.4	73.5

Form of PAS →	Tablet B						Tablet C						Tablet D					
Hours after ingestion →	2	4	6	8	24	32	2	4	6	8	24	32	2	4	6	8	24	32
Subjects:																		
1	10.1	45.7	59.9	70.3	77.0	78.1	1.6	19.0	33.8	52.2	59.6	62.2	0.4	25.0	41.0	53.4	75.0	76.0
2	8.5	38.7	61.0	66.1	71.4	71.9	2.4	38.2	52.0	59.0	69.0	74.0	8.4	49.4	64.0	68.2	72.2	74.4
3	11.2	35.9	55.3	65.0	75.9	78.3	3.2	35.4	54.8	64.0	79.0	84.8	27.2	57.6	62.8	67.8	76.6	79.6
4	3.7	32.1	58.5	68.5	74.8	76.5	1.4	16.0	61.8	63.4	69.6	70.8	0.8	14.6	50.0	61.8	72.2	74.0
5	15.3	44.9	53.4	56.5	58.9	60.1	4.0	46.0	58.8	66.0	73.4	75.0	1.4	29.6	60.0	66.2	73.8	76.2
6	4.0	45.0	56.1	60.1	67.3	67.6	2.0	32.2	65.8	63.4	67.8	69.0	1.6	42.0	55.8	61.6	68.6	69.8
7	9.9	46.7	58.6	62.3	66.8	67.4	8.0	52.4	53.8	70.4	72.2	73.4	3.0	36.2	56.0	61.6	64.0	66.6
Mean →	8.9	41.2	57.5	64.1	70.3	71.4	3.2	34.2	54.4	62.6	70.0	72.7	6.1	36.3	55.7	62.9	71.8	73.8

Form of PAS →	Tablet E						Tablet F						Tablet G					
Hours after ingestion →	2	4	6	8	24	32	2	4	6	8	24	32	2	4	6	8	24	32
Subjects:																		
1	0.3	8.2	18.9	41.8	64.4	66.3	0.0	0.0	1.4	5.7	33.0	45.1	0.2	0.5	0.8	0.8	0.8	1.4
2	0.1	3.6	9.5	15.9	50.4	51.9	0.0	0.5	8.6	29.5	57.6	61.9	0.3	0.5	0.6	0.6	2.3	8.9
3	1.0	6.9	20.0	43.9	70.8	74.1	0.5	1.4	5.2	15.6	49.2	56.9	0.5	0.5	0.6	0.8	1.7	1.7
4	0.3	7.6	17.7	28.1	53.3	55.5	0.3	0.5	12.2	34.6	58.9	63.8	0.6	0.9	1.2	1.4	2.8	3.7
5	0.6	16.6	41.1	55.5	68.3	70.7	0.8	4.1	7.0	9.5	45.4	57.8	0.3	0.6	0.9	0.9	4.0	19.7
6	0.1	5.2	26.9	38.8	63.4	64.8	0.0	0.0	0.5	3.2	45.7	54.9	1.2	1.2	1.4	2.0	2.9	4.1
7	0.4	9.2	22.6	47.3	56.3	57.9	0.3	0.3	1.6	10.8	54.6	57.8	0.6	1.1	1.4	1.7	2.4	6.6
Mean →	0.4	8.2	22.4	38.8	61.0	63.0	0.3	1.0	5.2	15.6	49.2	56.9	0.5	0.8	1.0	1.2	2.4	6.6

not significantly different from the standard at any time period. The excretion from tablets, B and C was highly significantly different from the standard for the two- and four-hour periods, significantly different at the six-hour period but not at the eight-, twenty-four-, and thirty-two-hour periods. The excretion from Tablet D was highly significantly different from the standard for the two-, four-, and six-hour periods, just reached significance at the eight-hour period, and was not significantly different for the twenty-four- and thirty-two-hour periods. The excretion of Na PAS following the ingestion of tablets E, F, and G was highly significantly different from the standard for all time periods. In view of the amounts of Na PAS excreted in the twenty-four-hours following the ingestion of the tablets as compared with the standards, it was felt that tablets A, B, C, and D were satisfactory from the standpoint of physiological availability while tablets E, F, and G were unsatisfactory. It is of interest to note that tablets E, F, and G would also have been considered unsatisfactory if based on the eight-hour excretion. That is, the eight-hour excretion of Na PAS from tablets A, B, C, and D was not significantly different from the standard but the eight-hour excretion from tablets E, F, and G was significantly different. In this case as in the results of the riboflavin study (4) the eight-hour excretion resulted in the same conclusion regarding availability as was arrived at using the twenty-four-hour excretion values.

As can be seen from the data in Table II, the coefficients of variation of the excretions of seven individuals following the ingestion of the standards and tablets A, B, C, and D are relatively small and uniform not only between the standards and tablets but between the eight- and twenty-four-hour periods. The excretions from tablets E, F, and G, however, had large coefficients of variation and less agreement between the eight- and twenty-four-hour periods. This variation indicated that the Na PAS in the latter tablets was not available to some subjects.

Disintegration Test.—Because of the fact that simulated gastric and intestinal fluids are listed in U. S. P. XV (7) it was considered desirable to compare them with the procedure used by Chapman, Crisafio, and Campbell (4) which employed rubber disks. Plastic disks were used in conjunction with the U. S. P. fluids and have the advantage that they remain horizontal while in motion and afford better visibility than do the rubber disks.

The mean *in vitro* disintegration times for the seven tablets as determined by both procedures are shown in Table III. There was a significant difference in the times between the two methods for tablets, A, B, F, and G, the method of Chapman, *et al.* (4), giving slightly longer times for tablets A and B, but a shorter time for tablets F and G. In the disintegration test of Chapman, *et al.*, tablet E had a disintegration time of 84.4 minutes and tablet F a time of 76.1 minutes and yet the Na PAS from tablet F was excreted to a smaller extent than from tablet E. When these two tablets were disintegrated using the U. S. P. fluids and the plastic disks, tablet E was found to have a disintegration time of 91.1 minutes and tablet F 111.5 minutes. These times were consistent with the per cent excretions from these two tablets as shown in Fig. 1. These results suggested that the use of the U. S. P. XV media and plastic disks gave a truer indication, in this one instance, of physiological availability than did the procedure of Chapman, *et al.* (4). Crisafio, Taylor, and Chatten (9) in comparing the media used by Chapman, *et al.* (4), and that given in the U. S. P. XV, found that the difference in disintegration times between the two media was caused by the alkaline rather than by the gastric fluid. This might be partially explained by the differences in *p*H and composition of the two alkaline media. Tablets E, F, and G were found to remain intact for a total of sixty minutes when tested in gastric juice alone.

In order to determine at what rate the Na PAS was being dissolved out of the tablets during the *in vitro* disintegration test, aliquots from the media as used by Chapman, *et al.* (4), were drawn off at ten-minute intervals and assayed. Five typical curves showing the rate at which the Na PAS is released into the disintegration media are shown in Fig. 2. The time shown is the total disintegration time which includes thirty minutes in the simulated

TABLE II.—COEFFICIENTS OF VARIATION OF THE EXCRETIONS OF NA PAS BY SEVEN INDIVIDUALS

Form of Na PAS	Period	
	8-hour	24-hour
Solution of PAS	±9.7%	±8.3%
Capsules of PAS	6.9	5.7
Tablet:		
A	5.6	6.3
B	7.5	9.1
C	9.1	8.4
D	8.1	5.8
E	33.8	12.8
F	24.4	18.3
G	44.2	41.7

TABLE III.—MEAN DISINTEGRATION TIMES OF SEVEN TABLETS BY TWO METHODS ON THREE SEPARATE DAYS

Tablet	Disintegration Time in Minutes			
	Chapman, *et al.*	Mean	Vliet, U. S. P. XV[a]	Mean
A	26.2	24.0	19.5	19.5[b]
	22.6		19.3	
	23.2		19.8	
B	66.2	65.6	53.0	58.0[b]
	64.3		64.8	
	66.3		56.3	
C	64.3	61.8	67.6	67.0
	66.0		72.8	
	55.0		60.8	
D	65.6	65.9	66.8	63.6
	64.8		59.2	
	67.5		64.8	
E	84.8	84.4	92.0	91.1
	90.0		83.8	
	78.5		97.5	
F	79.1	76.1	94.8	111.5[b]
	72.1		133.5	
	77.0		106.3	
G	131.0	134.5	197.3	200.4[b]
	133.8		210.0	
	138.6		194.0	

[a] Using plastic disks as described on page 374.
[b] Difference between methods is significant at P = 0.05.

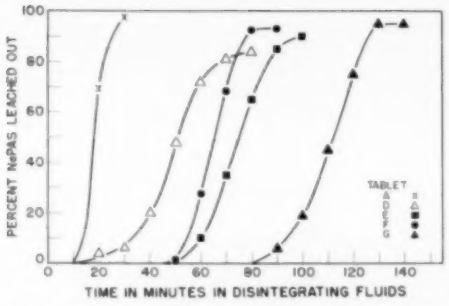

Fig. 2.—Relationship between time in disintegrating fluids and per cent of Na PAS leached out into media of Chapman, *et al.*

gastric juice and the balance in the simulated intestinal juice. These rates of release *in vitro* reflect the rates of release *in vivo* as shown in Fig. 1.

Relation Between Physiological Availability and Disintegration Time.—The relationship between disintegration time and the excretion of Na PAS from the tablets expressed as a percentage is shown in Fig. 3. Those tablets having disintegration times of approximately sixty-seven minutes or less were considered to be available while those having longer disintegration times were not completely available. In Fig. 3 a line has been drawn arbitrarily at the seventy-minute mark to separate those which were considered to be available from those which were unavailable.

It can also be seen in Fig. 3, that for those tablets having disintegration times of sixty-seven minutes or less the amounts of Na PAS excreted in the eight- and twenty-four-hour periods are quite similar. The same was found to be the case for tablet G having a very long disintegration time. However, for tablets E and F there was a wide difference between eight- and twenty-four-hour excretions.

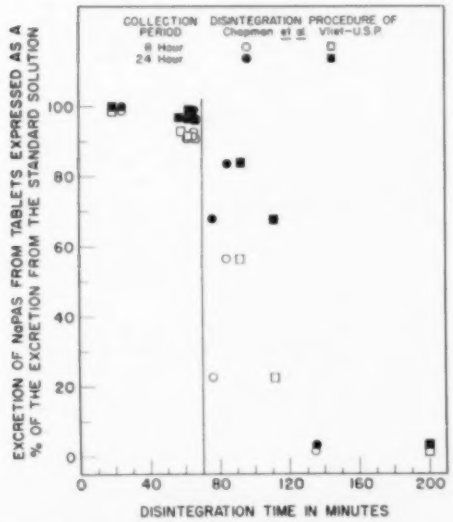
COLLECTION PERIOD
8 Hour
24 Hour
DISINTEGRATION PROCEDURE OF
Chapman et al.
Vitel—U.S.P
EXCRETION OF NaPAS FROM TABLETS EXPRESSED AS A % OF THE EXCRETION FROM THE STANDARD SOLUTION
DISINTEGRATION TIME IN MINUTES

Fig. 3.—Relationship between disintegration time and physiological availability of Na PAS.

DISCUSSION

The use of Na PAS has been found to lend support to the relationship between *in vitro* disintegration time and physiological availability which was established using riboflavin as a marker. In the present study, those tablets having disintegration times of approximately sixty-seven minutes or less were considered to have satisfactory physiological availabilities. In the riboflavin study (4), those tablets having disintegration times of sixty minutes or less were found to be satisfactory, while two tablets having disintegration times of sixty-five minutes were quite unavailable. This would suggest that tablets having *in vitro* disintegration times between sixty and seventy minutes, may or may not be physiologically available. In view of this and in order to ensure that all tablets are completely available, it would appear to be desirable to retain the sixty-minute time as being the maximum for a tablet that is to be satisfactory. As was found when using riboflavin, an eight-hour collection of urine gave, with but one exception, as accurate an indication of availability as the longer collection period. This fact is of considerable practical significance when human subjects are involved.

The smaller twenty-four-hour excretion (72.5%) of Na PAS found in the present study as compared with the 81–85% reported by Way, *et al.* (6, 10), may be accounted for by the fact that only 500 mg. was ingested as compared with 4 Gm. in the studies by Way, *et al.* Of the total Na PAS found in the urine, 9.4% was in the free form and 90.6% in the conjugated form; however, Way, *et al.* (6), found that approximately 60% was present in the conjugated form. The higher percentage of the conjugated form found in the present study is consistent with the findings of Zu Schwabedissen and Zimmerman (11), who have found that the percentage of conjugated *p*-aminosalicylic acid excreted decreases with increasing dose.

The relationship between the rate at which the medicaments of a tablet are leached out into the disintegrating media and the rate at which they appear in the urine was considered to be of interest. It was noted that there was a time lag before tablets A, B, C, and D started to disintegrate both *in vitro* and *in vivo*, and it would appear that the rate of release *in vivo* as judged by urinary excretion is roughly proportional to the rate of release *in vitro* as judged by the assay of the aliquots of the disintegrating media. Thus a tablet disintegrating slowly throughout the sixty-seven-minute period *in vitro* would apparently gradually release all of its medicament *in vivo* during the total time that the tablet is available to the body.

In the present study, the authors have not concerned themselves with the levels of Na PAS which might have appeared in the blood. However, mention might be made of two reports which have appeared in the literature dealing with blood levels of *p*-aminosalicylic acid. As a result of their work, Duncan, *et al.* (12), reported that there was little difference in the blood levels attained after using either the free acid or the sodium salt of PAS, but

that compressed tablets or enteric-coated capsules gave lower blood concentrations but maintained this level for a longer period of time. These authors have stated that this was apparently a result of delayed rather than incomplete absorption of the drug. Undoubtedly the delayed absorption was a reflection of slower disintegration. Way, *et al.* (6), have shown that following the ingestion of PAS, peak plasma levels occur between the first and second hour. In the present study it has been found that the peak urinary levels occur at the two-hour period. Not only does this suggest that PAS is rapidly excreted following its appearance in the blood stream, but also that the level found in the urine might actually give an indication of the level attained in the blood.

While all the tablets investigated in this study were sugar-coated, it might be assumed that enteric-coated tablets, to be quantitatively available, would also have to meet the *in vitro* disintegration test.

Since this paper was written, O'Brien, Pacenti, and Duescher have published the results of a study on tablet disintegration using a modified plastic disk (13). These disks were found to have the following advantages: (*a*) they are transparent, permitting continuous observation of the state of disintegration, (*b*) they ride smoothly and uniformly in the tubes, and (*c*) their design is simple, making them easy to use and clean. The results of O'Brien, *et al.* (13), have confirmed earlier work reported from this laboratory (4) in that the use of a movable disk is necessary to obtain more uniform results in the disintegration technique.

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The *in Vitro* Antibacterial Activity of Oils*

By JASPER C. MARUZZELLA† and MARVIN B. LICHTENSTEIN‡

A reinvestigation and evaluation of the *in vitro* antibacterial activity of oils (volatile, terpeneless, fixed, and infused) was carried out by using the filter paper disk method. The oils were tested against various pathogenic and nonpathogenic Gram-positive and Gram-negative bacteria.

THE ANTIBACTERIAL activity of oils and their constituents has been known for many years. The methods used for ascertaining this activity were essentially the phenol coefficient method, (1–6), turbidimetric, and dilution methods (7, 8). More recently the agar cup-plate method and filter paper disk method have been used (9, 10). This investigation was undertaken to re-evaluate the *in vitro* antibacterial activity of oils using the filter paper disk method exclusively.

MATERIALS AND METHODS

The detection of the antibacterial activity of various volatile oils, terpeneless oils, fixed oils, and infused oils was made by observing their effects on growing cultures of ten organisms. The organisms used were: *Neisseria perflava*, *Sarcina lutea*, *Bacillus mesentericus*, *Aerobacter aerogenes*, *Bacillus subtilis*, *Micrococcus pyogenes* var. *aureus*, *Pseudomonas aeruginosa*, *Serratia marcescens*, *Escherichia coli*, and *Proteus vulgaris*. Twenty-four-hour cultures of these organisms were grown in nutrient broths. Stock cultures were subcultured on fresh agar slants every seven days.

The method used for determining the presence or absence of antibacterial activity was a slight modification of Vincent's (11) qualitative filter paper disk diffusion plate method. In this method small sterile disks of filter paper were thoroughly moistened in the oil to be tested and then placed on the nutrient agar plates which had been previously seeded with 2 cc. of a twenty-four-hour broth culture of the organism. The plates were incubated at 37° for twenty-four hours. The presence of a definite zone of inhibition of any size surrounding the paper disks indicated antibacterial activity.

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TABLE I.—INHIBITORY ACTIVITY OF VOLATILE OILS

Oils	Zone of Inhibition in mm.[a]										
	N. perflava	*S. lutea*	*B. mesentericus*	*Aerobacter aerogenes*	*B. subtilis*	*M. aureus*	*Pseudomonas aeruginosa*	*Serratia marcescens*	*E. coli*	*P. vulgaris*	Sum[b]
Amber, Rect.	0	3	2	0	3	2	0	2	0	2	14
Amyris balsamifera	0	3	2	4	0	0	0	0	0	0	9
Angelica root	0	0	3	0	10	3	0	0	0	3	19
Angelica seed	0	2	2	0	4	4	0	0	0	3	15
Anise	1	0	3	0	4	0	0	1	0	1.5	10.5
Balsam peru	2	4	2	1	4	2	0	4	2	4	25
Balsam tolu	0	10	4	3	7	4	2	4	4	8	46
Bay	0	7	5	0	11	0	2	5	0	0	30
Bay laurel	0	0	0	0	24	0	0	3	0	0	27
Bergamot	0	5	6	0	10	0	3	8	3	0	35
Birch tar	5	13	7	3	8	5	5	14	6	12	78
Bitter orange, Italian	0	0	0	0	4	0	0	0	0	2	6
Black pepper	0	3	0	0	0	0	0	0	0	0	3
Calamus	0	3	0	0	3	0	0	0	0	1	7
Cananga	0	2	0	0	2	0	0	0	0	2	6
Caraway	0	0	2	0	9	0	2	2	0	0	15
Cardamom	0	0	2	0	10	0	0	0	0	0	12
Cascarilla	0	15	2	0	4	0	0	0	0	3	24
Cedar leaf	4	0	0	3	11	0	4	11	7	6	46
Celery seed	0	0	0	0	8	0	0	0	0	3	11
Chamomile, English	0	0	2	0	0	0	0	0	0	0	2
Chenopodium	0	0	3	0	6	2	0	3	0	8	22
Cinnamon	0	6	16	0	21	0	4	0	5	12	64
Citronella, Ceylon	0	0	3	0	8	0	0	0	0	0	11
Clove	0	4	4	0	7	0	0	0	0	2	17
Coriander	0	0	4	0	11	0	0	3	0	0	15
Cubebs	0	3	0	0	1	0	0	0	0	0	4
Cumin	0	0	15	0	15	0	0	2	0	0	32
Cypress	0	0	5	0	4	0	0	0	0	3	12
Dill seed	0	0	0	0	10	0	0	2	2	0	14
Dill weed	0	0	0	0	15	0	0	0	0	0	15
Dwarf pine needles	0	0	0	2	5	3	0	0	0	5	15
Eucalyptol	0	2	4	0	6	0	0	3	4	10	29
Eucalyptus	4	4	10	0	34	0	4	7	3	17	83
Fennel	0	0	2	0	4	0	0	0	0	0	6
Fir needle, Canadian	0	0	0	0	10	0	0	0	0	3	13
Fleabane	0	0	2	0	2	0	0	0	0	1	5
Geranium, Algerian	0	0	3	0	8	0	0	1	0	2	14
Ginger	0	2	2	0	2	0	0	0	0	2	8
Gingergrass, imported	0	0	5	0	15	0	0	3	2	0	25
Hemlock	1	3	10	0	10	2	0	2	0	5	33
Juniper berries	0	5	3	0	3	3	0	4	1	10	29
Juniper tar	0	4	2	0	4	0	0	0	0	2	12
Juniper wood, comp.	0	0	0	0	4	0	0	0	0	1	5
Laurel leaves, dist.	0	0	0	0	0	0	0	3	0	0	3
Lavender	2	0	4	2	17	3	0	2	0	0	30
Lavandin, French	0	0	3	0	5	3	0	3	0	0	14
Lemon, exp., American	0	0	4	0	5	0	0	0	0	0	9
Lemongrass	0	0	0	0	0	0	3	2	0	0	5
Lime, dist., natural, West Indian	0	0	4	0	4	0	0	1	0	0	9
Lime, exp., natural West Indian	0	0	5	0	3	0	0	0	0	0	8
Linaloe wood	0	0	2	0	25	0	0	0	0	0	27
Mace, dist.	0	0	0	2	10	0	0	0	0	0	12
Mandarin	0	15	3	1	3	0	0	0	0	9	31
Marjoram	2	0	3	0	10	3	0	2	3	0	23
Neroli	2	0	7	0	19	0	0	0	2	2	32
Niobe	0	0	0	0	20	0	0	0	0	0	20
Origanum, white	0	0	10	0	0	0	2	0	0	0	12
Orris root	0	2	2	0	2	0	0	0	0	2	8
Parsley seed, imported	0	4	3	3	4	2	4	4	2	4	30
Patchouly	0	1	0	0	1	0	0	0	0	0	2
Pennyroyal	2	10	3	1	10	2	0	3	2	0	33
Peppermint	2	5	3	0	8	3	0	2	3	0	26
Petitgrain, Paraguay	0	0	3	0	12	0	0	0	0	4	19
Pimento	0	2	3	0	9	0	0	2	0	0	16
Pimento leaf	0	6	4	0	8	3	0	4	0	6	28

TABLE I.—*Continued*

Oils	Zone of Inhibition in mm.[a]										
	N. perflava	*S. lutea*	*B. mesentericus*	*Aerobacter aerogenes*	*B. subtilis*	*M. aureus*	*Pseudomonas aeruginosa*	*Serratia marcescens*	*E. coli*	*P. vulgaris*	Sum[b]
Pine	3	0	6	3	4	3	0	3	0	0	22
Pine needle	2	0	0	0	3	0	0	2	0	5	12
Pine needle, Siberian	0	0	0	0	10	0	0	0	0	3	13
Pinus sylvestris	3	0	2	0	3	2	0	0	1	4	15
Rectified tar	0	0	0	0	17	0	7	0	0	0	24
Rhodium, ordinary	0	2	0	0	2	0	0	0	0	0	4
Rose	0	1	3	0	3	0	0	0	0	2	9
Rosemary	3	0	3	3	8	2	2	4	3	0	28
Rue	0	0	2	0	2	0	15	0	0	0	19
Sage	0	10	0	1.5	0	0	0	2	0	0	13.5
Sage, Grecian	0	0	0	0	10	3	0	0	0	0	13
Sage, Spanish	0	0	0	0	0	0	0	4	0	0	4
Sandalwood	0	2	2	0	4	0	0	0	0	0	4
Sassafras	0	0	2	0	6	1	0	2	0	0	11
Savin	0	0	3	0	5	2	0	0	0	3	13
Spearmint, Mohawk	0	0	4	0	5	0	0	4	0	0	13
Spike lavender	0	0	5	2	4	0	0	2	2	0	15
Sweet basil, American	0	0	3	0	12	0	0	3	0	0	18
Sweet birch	0	0	2	0	5	0	0	0	0	0	7
Sweet orange, exp., American	0	0	2	0	4	0	2	0	0	0	8
Sweet orange, exp. Italian	0	0	2	0	2	0	2	0	0	0	6
Tansy	0	0	0	2	20	0	2	3	1	0	28
Tarragon	0	0	0	0	10	0	0	3	0	0	13
Terpineol	0	0	3	0	8	0	0	4	3	0	18
Thuja occidentalis	0	4	3	0	5	0	0	3	0	0	15
Thyme	3	0	0	0	25	0	0	4	4	0	36
Thyme, red	0	0	0	0	25	0	1	4	0	0	30
Turpentine	2	3	3	0	3	3	0	0	3	5	22
Valerian, imported	2	5	3	1	5	2	0	2	2	4	26
Verbena, comp.	0	0	3	0	17	0	0	2	0	0	22
Vetivert, Haitian	0	3	2	0	4	0	0	0	0	0	9
Wintergreen, northern	0	0	2	0	0	3	2	2	0	0	9
Wormwood	0	0	3	0	12	0	0	0	0	0	15
Ylang-Ylang	0	0	2	0	8	0	0	0	0	0	10

[a] Measurement from disk edge to zone edge. [b] Sum of zones of inhibition for each oil. [c] Zone of inhibition absent.

The zone of inhibition was measured to 0.5 mm. by means of a metric ruler and a magnifying reading glass. The magnifying glass was suspended at a proper focal length of the lens above the illuminated plates. Plates were illuminated from below with a 75-watt incandescent light bulb. As the oils arrived at the laboratory they were immediately placed into sterile vaccine bottles. All of the oils were tested for sterility by streaking on nutrient agar slants and incubating at 37° for one to three days.

RESULTS AND DISCUSSION

Of the 110 volatile oils tested, 100 were found to exhibit antibacterial activity on at least one of the 10 organisms employed. Table I lists those oils with antibacterial activity and their zones of inhibition. Table II shows the antibacterial activity of 11 terpeneless oils. No attempt was made to ascertain whether the antibacterial activity of the oils listed in Tables I and II was bactericidal or bacteriostatic. From Tables I and II it can be observed that the terpeneless oils possess a less powerful inhibiting activity than the corresponding volatile oil. Those volatile oils which were found to possess no antibacterial properties are listed in Table III. The eight fixed oils and six infused oils used in this investigation exhibited no antibacterial properties. These oils are listed in Table III also. All of the oils used in this investigation were found to be bacteria free by streaking on agar slants.

From Table I the effect of the volatile oils against each organism was found to be of the following order (figures indicate the sum of the zones of inhibition caused by the oils): *Bacillus subtilis*, 767 mm.; *Bacillus mesentericus*, 268 mm.; *Proteus vulgaris*, 186.5 mm.; *Sarcina lutea*, 178 mm.; *Serratia marcescens*, 165 mm.; *Micrococcus pyogenes* var. *aureus*, 70 mm.; *Escherichia coli*, 70 mm.; *Pseudomonas aeruginosa*, 68 mm.; *Neisseria perflava*, 45 mm.; and *Aerobacter aerogenes*, 37.5 mm. Thus *B. subtilis* was the most vulnerable while *Aerobacter aerogenes* was the most resistant. From Table I it may be observed that the following oils show the greatest sum total of zones of inhibition in this order: eucalyptus, birch tar, cinnamon, balsam of tolu and cedar leaf. Furthermore the following volatile

TABLE II.—INHIBITORY ACTIVITY OF TERPENELESS OILS

Oils	Zone of Inhibition in mm.[a]										
	N. perflava	*S. lutea*	*B. mesentericus*	*Aerobacter aerogenes*	*B. subtilis*	*M. aureus*	*Pseudomonas aeruginosa*	*Serratia marcescens*	*E. coli*	*P. vulgaris*	Sum[b]
Anise	0[c]	0	0	0	8	0	0	2	0	0	10
Bay	0	3	4	0	5	3	0	4	0	0	19
Bergamot	0	0	4	0	18	0	0	0	0	3	25
Caraway	0	0	0	0	2	0	0	4	3	0	9
Cinnamon	0	2	15	0	15	0	5	11	0	0	48
Eucalyptus	0	3	0	0	0	0	0	3	0	0	6
Mandarin	0	0	0	0	10	0	0	2	0	0	12
Peppermint	2	0	0	2	4	2	0	2	0	0	12
Petitgrain	0	0	2	0	3	0	0	0	0	2	7
Sweet orange	0	0	0	0	0	0	0	2	0	0	2
Spearmint	0	0	3	0	0	0	0	3	3	0	9

[a] Measurement from disk edge to zone edge. [b] Sum of zones of inhibition for each oil. [c] Zone of inhibition absent.

TABLE III.—OILS WITH NO INHIBITORY ACTIVITY

Volatile Oils:	Cajuput	Myrrh
	Copaiba	Niaouli
	Garlic	Nutmeg, East Indian
	Hops	Origanum, red
	Laurel, exp.	Savory, select
Fixed Oils:	Castor	Croton
	Chaulmoogra	Olive
	Coconut	Persic
	Cod liver	Sweet almond, exp.
Infused Oils:	Asafetida	Lobelia
	Burdock	Mullein
	Henbane	Skunk

oils were found to be effective against eight or more organisms in the following order: birch tar, balsam of peru, balsam of tolu, parsley seed, valerian, eucalyptus, pennyroyal, and rosemary.

The negative results obtained with fixed oils do not agree with the work of Bello (12) and Lembach (13) who found that castor oil and cod liver oil possess antibacterial properties against staphylococci and *E. coli*. Since these investigators did not employ the filter paper disk method in their work the negative results here obtained may be explained in terms of fixed oils possessing a poor power of diffusion.

SUMMARY

1. The antibacterial activity of 110 volatile oils, 11 terpeneless oils, eight fixed oils, and six infused oils was tested against 10 different bacteria with the filter paper disk method.

2. The volatile oils which exhibited the greatest antibacterial activity were: eucalyptus, birch tar, cinnamon, balsam of tolu, and cedar leaf.

3. The following volatile oils were found to possess no antibacterial activity: cajuput, copaiba, garlic, hops, laurel (expressed), myrrh, niaouli, nutmeg (East Indian), origanum (red), and savory (select).

4. Terpeneless oils have a less powerful antibacterial activity than the corresponding volatile oil.

5. The fixed and infused oils possess no antibacterial properties.

6. *Bacillus subtilis* was shown to be the most susceptible organism to the volatile oils while *Aerobacter aerogenes* was the most resistant.

7. The following volatile oils were effective in inhibiting eight or more of the 10 organisms employed: birch tar, balsam of peru, balsam of tolu, parsley seed, valerian, eucalyptus, pennyroyal, and rosemary.

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Effect of Alteration of Size of the Aromatic Dicarboxylic Acid Nucleus on Local Anesthetic Activity*

By WILLIAM C. NORTH and KARL F. URBACH†

Dialkylaminoalkyl esters of 4,4′-diphenyl propanedicarboxylic acid; 4,4′-dibenzyldicarboxylic acid; 4,4′-diphenylmethanedicarboxylic acid; 4,4′-diphenyldicarboxylic acid, and terephthalic acid were prepared as the hydrochlorides and tested for local anesthetic activity on the rabbit cornea and by intradermal injection in the guinea pig. Acute toxicity was studied by intraperitoneal and intravenous injection in mice. No predictable relationship between local anesthetic activity and the size of the dicarboxylic acid nucleus was evident. Toxicity decreased as the methylene groups between the benzene rings was reduced. Two esters appeared to have greater local anesthetic activity and lower toxicity than procaine.

IN THE REPEATED ATTEMPTS to correlate local anesthetic activity with chemical structure the relationship of the configuration of the alkylamino alcohol to relative potency has been established. The effect of alteration of the carboxylic acid nucleus on activity is not so clear (1–3).

Double bond conjugation in the acid radical is apparently an important factor in causing local anesthesia (4–11). Fosdick and Urbach synthesized esters of 4,4′-stilbenedicarboxylic acid and 4,4′-dibenzyldicarboxylic acid and found that the additional unsaturation of the stilbene compounds failed to make them more active than the dibenzyl analogs (12).

Because the dibenzyl esters seemed to possess desirable characteristics as local anesthetic agents (13) this study was undertaken to determine whether modification of the aliphatic carbon chain between the phenyl groups of the aromatic dicarboxylic acid nucleus would influence local anesthetic activity.

EXPERIMENTAL

Chemistry.—Previously unsynthesized dialkylaminoalkyl esters were prepared by converting the acids to the corresponding acid chlorides which were then esterified with the appropriate dialkylaminoalcohols.

Terephthalic acid was obtained from the Eastman Kodak Company. Dibenzyldicarboxylic acid was prepared as previously described (12). 4,4′-Diphenylmethanedicarboxylic acid was prepared according to the method of Lieberman using diphenyl methane and oxalyl chloride (14); yield-85%, m. p. 334°.

4,4′-Diphenyldicarboxylic acid was synthesized by the oxidation of 4,4′-diacetyldiphenyl, since attempts to prepare it by hydrolysis of the dicyanide (15) were unsuccessful. The 4,4′-diacetyldiphenyl was prepared by the method of Dilthey (16) giving a yield of 10%. The 4,4′-diacetyldiphenyl was oxidized by the method described by Koelsch for the preparation of terephthalic acid (17). The acid, obtained in a yield of 85%, did not melt below 350°.

The acid chlorides were prepared by the method previously used (14) with the modification that phosphorus pentachloride (which was found to materially increase the speed of the reaction) was added to the thionyl chloride. The physical characteristics of the acid chlorides are given in Table I.

The α-methylpiperidino-propanol was prepared by the method of McElvain and Carney (18). The other dialkylamino-alcohols were obtained from the Eastman Kodak Company.

The esters were prepared by refluxing one molecular weight of the acid chloride with four molecular weights of the dialkylamino-alcohol in anhydrous benzene (12). In each case the ester was precipitated as the hydrochloride by the addition of absolute ethanol saturated with dry hydrogen chloride. The precipitated amino alcohol hydrochloride was filtered and purified by solution in absolute methanol and refluxing with activated charcoal. The esters were crystallized from absolute methanol. Their characteristics are given in Table II.

Pharmacologic Evaluation.—Local anesthetic potency, as determined by duration of anesthesia, was tested on the rabbit cornea and by intradermal injections in guinea pigs. The compounds listed in Table III were dissolved in 0.85% NaCl solution.

In the rabbit the technique of Schmitz and Loevenhart (with slight modifications) was used (19). Healthy adult rabbits, whose hair and lashes about the eyes had been closely clipped, were placed in a stock designed to hold their heads motionless. The presence of an active corneal reflex was determined. By pinching the lower lid to form a cup, the eye was bathed in a solution of the local anesthetic for one minute. The cornea was then tested at frequent intervals for presence of anesthesia, as indicated by loss of the blink reflex, when a rounded probe was placed against the cornea. After the onset of anesthesia, the cornea was tested once a minute until anesthesia had disappeared. Irritation produced by these compounds on the cornea

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TABLE I.—PREPARATION AND PROPERTIES OF DICARBOXYLIC ACID DICHLORIDES

Acid chlorides	Wt. Acid Chloride, Gm.	Wt. PCl_5, Gm.	Vol. $SOCl_2$, ml.	Yield, %	Crystallized from	m. p.
4,4′-Diphenylmethanedicarboxylic acid dichloride	5	10	30	47	Petroleum ether	94–96
4,4′-Diphenyldicarboxylic acid dichloride	10	17.5	50	57	Benzene	183–183.5
Terephthalic acid dichloride	10	15	150	57.5	Benzene	77–78

TABLE II.—PROPERTIES OF THE DICARBOXYLIC ACID DIALKYLAMINOALKYL ESTER DIHYDROCHLORIDES

Acid	Alcohol	Mol. Wt.	Yield,[a] %	m. p.	Nitrogen Analysis Calculated	Found
4,4′-Dibenzyldicarboxylic acid	Di-*n*-butyl amino-*n*-propanol	681.8	35	152–154	4.11	4.12
	α-Methylpiperidino-*n*-propanol	621.7	50	216–217	4.51	3.99
4,4′-Diphenylmethanedicarboxylic acid	Diethylaminoethanol	527.5	55	185–188	5.31	5.34
	Dimethylaminoethanol	471.4	55	230–232	5.94	6.03
4,4′-Diphenyldicarboxylic acid	Diethylaminoethanol	513.5	60	253–255	5.46	5.62
	Dimethylaminoethanol	457.4	50	115–118	6.13	5.96
	Di-*n*-butyl amino-*n*-propanol	553.8	45	184–185	4.29	3.97
Terephthalic acid	Diethylaminoethanol	437.4	70	215–220	6.42	6.63
	Dimethylaminoethanol	381.3	65	244–245	7.37	7.58
	Di-*n*-butyl amino-*n*-propanol	577.7	70	170	4.85	4.45
	α-Methylpiperidino-*n*-propanol	517.6	35	245	5.41	4.57

[a] Based on the acid chloride.

TABLE III

Compounds

1. Bis(diethylaminoethyl)-4,4′-diphenyl-1,3-propanedicarboxylate HCl[a]
2. Bis(dimethylaminoethyl)-4,4′-diphenyl-1,3-propanedicarboxylate HCl[a]
3. Bis(di-*n*-butylamino-*n*-propyl)-4,4′-diphenyl-1,3-propanedicarboxylate HCl[a]
4. Bis(diethylaminoethyl)-4,4′-dibenzyldicarboxylate HCl
5. Bis(dimethylaminoethyl)-4,4′-dibenzyldicarboxylate HCl
6. Bis(di-*n*-butylamino-*n*-propyl)-4,4′-dibenzyldicarboxylate HCl
7. Bis(α-methylpiperidino-*n*-propyl)-4,4′-dibenzyldicarboxylate HCl
8. Bis(diethylaminoethyl)-4,4′-diphenylmethanedicarboxylate HCl
9. Bis(dimethylaminoethyl)-4,4′-diphenylmethanedicarboxylate HCl
10. Bis(diethylaminoethyl)-4,4′-diphenyldicarboxylate HCl
11. Bis(dimethylaminoethyl)-4,4′-diphenyldicarboxylate HCl
12. Bis(di-*n*-butylamino-*n*-propyl)-4,4′-diphenyldicarboxylate HCl
13. Bis(diethylaminoethyl)terephthalate HCl
14. Bis(dimethylaminoethyl) terephthalate HCl
15. Bis(di-*n*-butylamino-*n*-propyl) terephthalate HCl
16. Bis(α-methylpiperidino-*n*-propyl) terephthalate HCl
17. Cocaine
18. Procaine

[a] Prepared by Dr. T. R. Blohm in the Department of Chemistry, Northwestern University Dental School. Present address: Biochemistry Section, Pharmacology Department, Wm. S. Merrell Co., Cincinnati, Ohio.

or conjunctiva was noted. The animals were used only once in six weeks. It was noted that there was little correlation between the duration of anesthesia produced by the same compound in the two eyes of any rabbit. Therefore, the results are given in Table IV, as mean duration of anesthesia ± standard deviation.

The duration of anesthesia upon intradermal injection was tested by the method of Rose (20). Guinea pigs which had been sedated by an intraperitoneal injection of 20 mg./Kg. of Nembutal® were freed of hair over the back. Three wheals on either side of the spine were then made in the shaved area. Each wheal was formed by the injection of 0.3 cc. of a 1.0% solution of the anesthetic. The boundary of the wheal was outlined with ink. Using an inductorium, an electrical stimulus, of sufficient intensity to cause a skin reflex or squealing when used in unanesthetized areas of the skin, was applied to the site of injection every minute to determine time of onset and duration of anesthesia. The animals were examined daily for at least three days to determine whether necrosis or other evidence of irritation would appear. Significant difference from the procaine controls was determined by the "T" test using paired comparisons.

While the duration of anesthesia produced by any compound varied from animal to animal, the duration of anesthesia in wheals made with the same compound in the same animal was remarkably constant. Consequently, each drug tested had its own procaine control in the same animal. For that

TABLE IV.—PHARMACOLOGIC EVALUATION

Compound No.	Rabbit Cornea, 2%		Guinea Pig Wheals		
	No. Trials	Av. Duration, Min.	No. Trials	Av. Duration, Min. Compd., 1%	Procaine, 1%
1	4	20.5 ± 7.7	8	82.9[a]	53.6
2	4	34 ± 16.8	8	64.0	53.6
3	1	3	2	56.5	30.5
4	8	31.2 ± 15.8	10	31.1	29.2
5	2	5 ± 0	8	15.6	21.3
6	2	0[a]		Insol. in 0.85% NaCl	
7	6	33 ± 6.7	8	181.3[a]	42.1
8	7	29.3 ± 12.5	10	71.1[a]	33.4
9	4	5.5 ± 1.5	9	62.0	33.9
10	8	5.6 ± 1.2	6	54.5	44.3
11	4	6.0 ± 3.5	6	22.0	44.3
12	2	0	8	127.3[a]	42.1
13	2	0	8	18.8	23.5
14	2	0	8	1.1[a]	23.5
15	2	31.5 ± 6.5	8	98.8[a]	26.3
16	4	0	8	26.1	26.3
17	40	33.5 ± 13			

[a] Significantly different from procaine controls.

reason each compound in Table IV has its own procaine value for comparison.

Toxicity was determined in mice and compared to procaine and cocaine. The drugs were given intraperitoneally and intravenously. The LD_{50} by both routes for each compound was determined according to the method of Dragstedt and Lang (21). The results are listed in Table V.

TABLE V

Compound No.	Toxicity in Mice, mg./Kg. i.p. No. Mice	i.p. LD_{50}	i. v. No. Mice	i. v. LD_{50}	Irritation
1	21	440	30	58.5	
2	20	472	16	70	
3	Insufficient Compound				
4	26	446	23	73	
5	14	675	19	71	+
6	21	465	17	28	
7	19	175	19	19	+
8	20	590	22	54	
9	12	710	15	87	+
10	21	550	16	54	
11	10	1100	26	175	
12	25	450	18	71	+
13	18	1735	24	110	
14	16	1500	17	155	
15	20	390	..	...	+
16	22	312	..	...	+
17	13	72	..	...	
18	70	195	29	45	

DISCUSSION

The principal purpose of this study was to determine how modification of a dicarboxylic acid nucleus of alkylaminoalkyl esters, by changing the number of $-CH_2-$ groups between the benzene rings, would affect local anesthetic activity. Secondarily it was hoped that alteration of the alkylaminoalkyl side chains might produce a compound suitable for clinical use.

As was to be expected, all of the compounds tested possessed some local anesthetic activity upon injection. The lack of topical anesthetic activity of the dibutylaminopropyl esters of all the acids except terephthalic acid was surprising, since the dibutylaminopropyl radical has been considered an active topical anesthetic moiety. Upon injection, these esters, and the α-methylpiperidinopropyl esters, produced more prolonged anesthesia than the diethylaminoethyl esters which in turn were longer acting than the dimethylaminoethyl esters.

No definite trend was evident relating duration of topical or intradermal anesthesia to the size of the acid nucleus within a series of the same ester. On injection the diphenylmethanedicarboxylic acid esters appear to be the most potent. The terephthalic acid compounds have little activity except for the dibutylaminopropyl ester. In fact, the terephthalic acid esters behave quite differently from the other dicarboxylic acid esters.

Toxicity in mice appears to be more closely related to the acid structure, decreasing regularly with reduction in molecular size. This trend is more apparent upon intraperitoneal than upon intravenous administration, suggesting that the smaller compounds are more poorly absorbed or more readily destroyed than the larger ones. All of the drugs produced convulsions in lethal doses with the typical picture of respiratory failure.

The diethylaminoethyl ester of diphenylmethanedicarboxylic acid and the dibutylaminopropyl ester of terephthalic acid both possess greater activity topically and on injection and less toxicity than procaine. Whether this superiority to procaine would be manifest on clinical trial cannot be predicted.

SUMMARY AND CONCLUSIONS

1. A series of esters of several dicarboxylic acids were synthesized and tested for local anesthetic activity in rabbits and guinea pigs and for toxicity in mice.

2. No predictable relationship between anes-

thetic activity and size of the dicarboxylic acid nucleus could be observed.

3. There is a constant decrease in toxicity in mice when the carbon chain between the phenyl groups is shortened.

4. The diethylaminoethyl ester of 4,4′-diphenylmethanedicarboxylic acid and the dibutylaminopropyl ester of terephthalic acid possess both sufficient anesthetic activity and low enough toxicity to suggest that they may be clinically useful.

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Paper Chromatography of Bile Acids II

By DAVID KRITCHEVSKY and R. F. J. McCANDLESS

A system for the paper chromatography of bile acids is described and the R_f values for a number of acids are reported.

SINCE OUR INITIAL ARTICLE on the paper chromatography of bile acids using basic solvents (1) several other publications have appeared on this subject. Sjovall (2) reported the separation of taurocholic, glycocholic, cholic, hyodesoxycholic, and desoxycholic acid using a system in which 70% acetic acid is the stationary phase and isopropyl ether-heptane 7:3 is the moving phase. He has since increased the number of solvent systems which may be used as the moving phase in achieving bile acid separation (3, 4). Beyreder and Rettenbacher-Daubner (5) obtained separation of the above-named acids excepting hyodesoxycholic and including dehydrocholic using a toluene-acetic acid-water system. One other group of workers has successfully carried out paper chromatographic separation of a wide variety of bile acids using basic solvents (6).

Our original report concerned itself with cholic, norcholic, desoxycholic, and dehydrocholic acids. This work has now been expanded to include bile acids of greater biological significance. The solvent systems used have been *n*-propyl alcohol-ammonia-water 90:2:8, *n*-propyl alcohol-ammonia-ethanolamine-water 89:2:1:8, and 90:1:3:6, respectively. All experiments were performed using 50γ of each acid.

For identification of the bile acids we used either 10% phosphomolybdic acid in absolute alcohol (7), 10% sulfuric acid in acetic anhydride (v/v) (8), or 50% antimony trichloride in glacial acetic acid (6). The colors obtained with the latter spray have been reported for all the acids used here except for hyodesoxycholic acid, which gave a violet color with the reagent.

Our results are summarized in Tables I and II.

EXPERIMENTAL

All solvents were distilled prior to use. Mixtures

TABLE I. R_f VALUES FOR BILE ACIDS

Acid	P[a]-A[b]-W[c] 90:2:8	P-A-M[d]-W 89:2:1:8	P-A-M-W 90:1:3:6
Cholic	0.56	0.57	0.91
Glycocholic	0.44	0.61	0.81
Taurocholic	0.55	0.50	0.74
Desoxycholic	0.73	..	0.87
Hyodesoxycholic	0.74	0.74	0.83
Lithocholic	0.83	0.87	..

[a] P = *n*-propyl alcohol. [b] A = ammonia. [c] W = water. [d] M = monoethanolamine.

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We are indebted to Professor Sune Bergstrom, Lund, Sweden, for the generous gift of pure tauro and glycocholic acids.

TABLE II. COLOR REACTIONS OF BILE ACIDS WITH SULFURIC ACID-ACETIC ANHYDRIDE REAGENT[a]

Acid[d]	5% H_2SO_4[b]		7.5% H_2SO_4		10% H_2SO_4		15% H_2SO_4	
	Visible	U. V.[c]	Visible	U. V.	Visible	U. V.	Visible	U. V.
Cholic	Violet	Pink	Violet	Pink	Violet	Pink	Violet	Pink
Glycocholic	Violet	Peach	...	Brick	Pink	Brick	Violet	Peach
Taurocholic	Violet	Pink	Violet	Brick	Violet	Red	Violet	Pink
Desoxycholic	...	Pink	...	Pink	...	Pink	Red	...
Hyodesoxycholic	...	...	...	...	...	...	Brick	...
Lithocholic	...	...	...	...	...	...	...	...

[a] Colors observed after heating air-dried papers at 100° for 2 minutes. [b] All solutions are v/v. [c] U. V. light of max. 366 mμ. [d] 50 γ of each acid used.

of solvents are given by volume. The filter paper used was Whatman No. 1.

The bile acid being chromatographed was applied to a spot 2 cm. in diameter on a 4 x 40 cm. strip of filter paper. Descending chromatography was employed and the solvent front was allowed to advance 25–30 cm. before the strip was removed from the glass chromatographic chamber and air dried. The strips were dried at 80° for fifteen minutes before spraying. The papers sprayed with phosphomolybdic acid were dried at 80°–90° for five minutes, those sprayed with antimony trichloride were dried at 95° for five minutes, and those sprayed with sulfuric acid-acetic anhydride were dried at 100° for two to three minutes.

The R_f values were measured from the foremost point of the origin to the leading edge of the spot. All R_f values represent the average of a number of experiments.

The chromatography was carried out at room temperature.

DISCUSSION

Examination of the data in Table I shows that while it is possible to distinguish taurocholic, glycocholic, and desoxycholic acids from cholic acid, to consider the compounds most often found in biological work (6, 9, 10), it is difficult to get distinct separation of the acids. Some differentiation can be obtained by observing the ultraviolet fluorescence of these compounds at 365 mμ after spraying with an antimony trichloride reagent. Carey and Bloch (11) have observed rust, yellow, rose, dark peach, and dark peach fluorescence for cholic, desoxycholic, lithocholic, taurocholic, and glycocholic acids, respectively. This method still leaves the two conjugated bile acids unresolved.

Prochazka (8) has reported on spray reagents for a variety of steroid acids, and several of these sprays seemed useful for our purposes. The 4% aq. potassium ferrocyanide, 4% aq. copper acetate, and 0.005% methanolic fluorescein sprays gave no colors with the bile acids. A 50% trichloroacetic acid spray showed nothing in the visible after drying at 100° for ten minutes and observed at 366 mμ, the first four acids listed in Table I showed a pale green fluorescence. The sulfuric acid-acetic anhydride reagent gave results which were interesting and useful. These are detailed in Table II.

SUMMARY

A technique for the paper chromatography of bile acids is presented and the R_f values for cholic, glycocholic, taurocholic, desoxycholic, hyodesoxycholic and lithocholic acids are given. A useful sulfuric acid-acetic anhydride spray reagent is described.

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The Colorimetric Determination of Peptic Activity*

By HAROLD J. WESSELMAN

An accurate and rapid colorimetric assay for peptic activity is proposed and compared with the N. F. IX assay. Hemoglobin is used as a substrate. The results obtained are easily reproduced.

THE ASSAY PROCEDURE described in the official monograph for pepsin in the National Formulary IX (1) has several disadvantages in so far as routine use is concerned. First, the time required for the assay amounts to approximately four hours overall and, secondly, the results obtained are sometimes questionable and quite often must be confirmed by repeated assay.

In a previous paper (2) a faster and more accurate procedure than that of the National Formulary IX (1) was described; however, this method requires the use of crystallized egg albumen as a substrate and its preparation is time consuming. Anson (3) described the use of hemoglobin for the estimation of pepsin. In this case the hemoglobin was obtained from fresh ox blood although mention is made of the fact that powdered hemoglobin could be used as a substrate with somewhat less accuracy. In the present work powdered hemoglobin is used and techniques have been developed which overcome any inaccuracy that may be due to its use.

An aqueous solution of hemoglobin is quite stable when kept refrigerated and has proved to be a uniform substrate. In this method the hemoglobin is digested by the pepsin under standard conditions at an optimum *p*H of 1.6–2.0. The undigested hemoglobin is precipitated with trichloracetic acid and removed by filtration. The hydrolytic products (polypeptids) are determined in the filtrate with the Folin phenol reagent (4) which gives a blue color due mostly to the tyrosine and tryptophane groupings. This reagent will react with as little as 1 p. p. m. of tyrosine.

EXPERIMENTAL

Reagents.—0.3% HCl: mix 35 cc. of 1 *N* hydrochloric acid with 385 cc. of distilled water; 0.5 *N* HCl; 0.5 *N* NaOH; N. F. reference pepsin: (dissolve 0.1 Gm. of N. F. reference pepsin in sufficient 0.3% HCl to make 150 cc.); hemoglobin substrate: (weigh accurately 25 Gm. of hemoglobin powder and add slowly with constant stirring to 900 cc. of distilled water contained in a one-liter beaker. Stir for a half-hour and transfer quantitatively to a one-liter volumetric flask. Dilute to volume with distilled water and store in a refrigerator. Difco Bacto-Hemoglobin has been found to be satisfactory for this purpose); 25% trichloracetic acid solution. Store in a refrigerator; phenol reagent (4).

Apparatus.—The apparatus used in this work consisted of a Beckman spectrophotometer, Model DU, matched corex absorption cells, 1-cm. light path.

Sample Preparation.—Transfer an accurately-measured aliquot of the sample equivalent to approximately 0.1 Gm. of 1:3000 pepsin to a 150-cc. volumetric flask. Add sufficient 0.3% HCl to make exactly 150 cc. and shake thoroughly. Use this dilution as the solution being assayed in the procedure below.

Procedure.—Transfer exactly 20 cc. of the hemoglobin substrate and 5 cc. 0.5 *N* HCl to each of three 100-cc. volumetric flasks marked "blank," "standard," and "unknown." To the flask marked standard add 5 cc. of N. F. reference pepsin and to the flask marked unknown add 5 cc. of the pepsin solution being assayed. Shake the contents of each flask and let stand for exactly 15 minutes. Then add 10 cc. of 25% trichloracetic acid solution to each flask, dilute to volume, and shake well.

Filter the contents of each flask through a dry filter paper, discarding the first few cc. of filtrate. Transfer exactly 10 cc. of each filtrate to separate 50-cc. volumetric flasks. Add to each flask 10 cc.

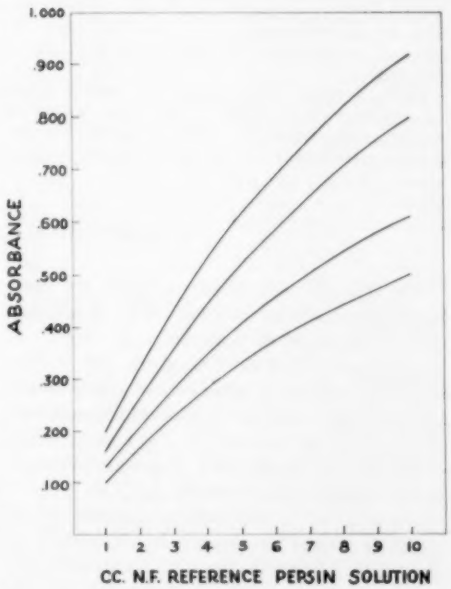

Figure 1.

* Received July 16, 1955, from the Analytical Department, Eli Lilly and Company, Indianapolis, Ind.

TABLE I.—COMPARISON OF RESULTS OBTAINED BY PROPOSED METHOD AND OFFICIAL METHOD

Product	Dilution		N. F. IX Method		Proposed Method Absorbance	
	Std.	Sample	Std. cc.	Sample cc.	Std.	Sample
Elixir lactated pepsin	1:3000	1:3000	2.5	2.5	0.640	0.650
	"	"	2.5	2.5	0.640	0.650
Essence pepsin	"	"	4.0	0.8	0.500	0.600
	"	"	4.0	0.8	0.500	0.600
Elixir enzymes aromatic	"	"	2.5	1.0	0.640	0.688
	"	"	2.5	1.0	0.640	0.688
Elixir pepsin compound	"	"	3.0	1.5	0.590	0.700
	"	"	3.0	1.5	0.590	0.700
Pepsin powder N. F.	"	"	2.5	2.0	0.640	0.650
	"	1:3500	2.5	3.5	0.640	0.610
Pepsin granular N. F.	"	1:3000	4.0	0.8	0.400	0.520
	"	1:3500	4.0	3.5	0.400	0.500
Pepsin powder 1:6000	"	1:6000	2.0	1.5	0.390	0.410
	"	1:7000	2.0	2.0	0.390	0.345
Pepsin granular 1:6000	"	1:6000	3.0	1.5	0.500	0.560
	"	1:7000	3.0	2.5	0.500	0.510

0.5 *N* NaOH and 1 cc. phenol reagent. Dilute to volume with distilled water and shake well.

Measure the absorbances relative to the reagent blank with a spectrophotometer at 700 mμ using 1-cm. matched corex cells. Absorbance of the reference pepsin can be compared to the unknown pepsin dilutions to determine the relative peptic activity of the unknown or absorbance can be converted to pepsin concentration with the aid of a calibration curve. The latter method is preferred when the exact amount of peptic activity needs to be determined.

RESULTS

Table I provides a comparison between the two methods which show good agreement.

Although the system does not obey the Lambert-Beer law, no difficulty is encountered as the N. F. reference pepsin is used as a standard in all determinations.

Figure 1 shows typical calibration curves which are easily set up. It will be noted that the curves are not congruent since the concentration of hemoglobin varies from lot to lot and the room temperature varies from day to day. In practice it has been found expedient to construct a new curve when needed rather than attempt to maintain precise control over the temperature during digestion and the concentration of the hemoglobin; however, the hemoglobin can be standardized on the basis of its iron or nitrogen content.

SUMMARY

The colorimetric method presented for the determination of peptic activity using hemoglobin as a substrate compares very favorably with the official assay. Accurate results are quickly obtained.

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SYMPOSIUM ON ANTIBIOTICS

The Fourth Annual Symposium on Antibiotics, sponsored by the Food and Drug Administration, Division of Antibiotics of the U. S. Department of Health, Education, and Welfare with the journals, *Antibiotics and Chemotherapy* and *Antibiotic Medicine & Clinical Chemotherapy* will be held on October 17, 18, and 19, 1956, at the Willard Hotel, 14th Street and Pennsylvania Avenue, N.W., Washington, D. C.

Contributors to the 1956 symposium are required to submit an abstract in triplicate of no more than 200 words, no later than September 3, 1956, and the original manuscript and one copy must be submitted by September 17, 1956.

Manuscripts must be on new, previously unpublished material. They must be typewritten, double spaced, and only on one side of 8½ x 11-inch paper. No more than six glossy photographs, no larger than 8½ x 11 inches, of all illustrations should accompany each manuscript. Bibliographies should conform to the style of the *Quarterly Cumulative Index Medicus*. Generic names of drugs should be used throughout the manuscript except that the first time the drug is mentioned the trade name may be referred to in a footnote. Send manuscripts to: Dr. Henry Welch, Director, Division of Antibiotics, Food and Drug Administration, Washington 25, D. C.

Glycolysis Inhibitors Among Compounds Containing the CON Structure

By R. S. MANLY and G. L. HARGREAVES

Over 400 organic substances possessing a CON grouping were studied for their ability to exert residual inhibitory action on glycolysis by oral microorganisms, when the organisms were present in a thin film resembling the dental plaque. Among amides inhibitory structures were frequently found for alkyl monoamides and lactamides containing 7 to 21 carbon atoms. Only two inhibitory substances were found among 154 carbamates, and none of 71 derivatives of urea were inhibitory when tested in aqueous solution.

PROBABLY A MAJORITY of investigators of the problem of dental caries agree with the hypothesis that this disease is initiated by dental plaques, which are accumulations of salivary components on teeth, including oral microorganisms. The thin layer of dental plaques is capable of forming surprising local concentrations of acid by glycolysis within ten minutes after contact with sugar solutions. The *p*H of plaques measured on the teeth of caries-susceptible persons has been shown to drop to 5.0 or less (16), which is sufficient to cause decalcification of tooth enamel (7). Several investigators of the problem of dental caries have sought to find inhibitors of glycolysis in hopes that such substance might prove to be of value in the reduction of dental caries (1–6, 8, 13–15).

An empirical survey of numerous chemicals has been in progress in this laboratory for four years. Nearly 2,500 chemicals have been studied for their ability to inhibit formation of acid from sugar by the microorganisms of saliva. The solutions of chemicals were tested under conditions resembling those which hold for dental plaques *in vivo*. Findings with certain simple organic structures have been reported. Active substances among phenols, alcohols, esters, and ethers have been presented orally (10), and results with an extended series of amines have appeared in print (11). The present work is concerned with over 400 compounds containing the CON grouping—amides, carbamates, and urea derivatives—with or without those previously evaluated, such as amines, acids, phenols, alcohols, ethers, or esters.

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The authors appreciate the cooperation of Columbia Southern Chemical Company, Chemical-Biological Coordination Center, Dow Chemical Company, Eastern Regional Laboratory, Jefferson, Chemical Company, Mallinckrodt Chemical Works, Dr. J. Phillip Mason of Boston University, and Sloan Kettering Institute, in furnishing ten or more compounds each. There were 27 other companies which supplied lesser numbers.

EXPERIMENTAL

The testing procedure (12) is based upon the centrifugate obtained from stimulated human saliva by centrifuging at 2,400 r. p. m. for twenty minutes. Thirty microliters of the sediment is held mechanically in a thin film against a glass electrode with the aid of a shaped thimble of nylon mesh. When an electrode coated in this manner is placed in a control buffer containing 0.01 molar bicarbonate and 0.2 molar glucose, the *p*H of the sediment falls and attains a steady state usually within thirty minutes. The *p*H differential between the buffer and the sediment at steady state is usually 1.5 to 2.5 units. This initial differential establishes the activity of the sediment samples and is the base for calculation of the effects of treatment. Next the control buffer is removed and replaced for twenty minutes by another sample of buffer to which the test chemical has been added. The concentrations employed are 0.1%, 1%, or half-saturation. Then the test solution is replaced for thirty minutes by a second control buffer. All throughout the test period, *p*H readings are recorded automatically every three minutes from the sediments and buffer solutions in three separate cells.

The effects were calculated from the change in *p*H differentials divided by the initial *p*H differentials (Fig. 1). The change from first to second period, divided by the first differential (times 100) indicates the effect during contact. The change from first to third, divided by the first differential (times 100) demonstrates the residual action of the chemical after rinsing in the buffer for thirty minutes. Residual inhibitory action requires that a chemical

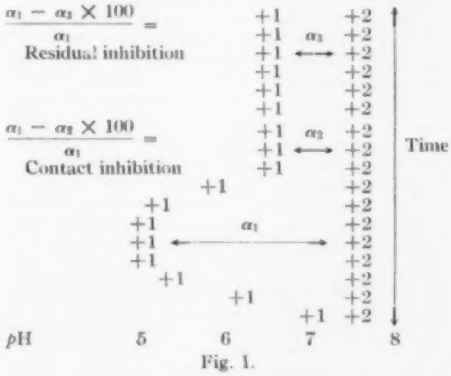

Fig. 1.

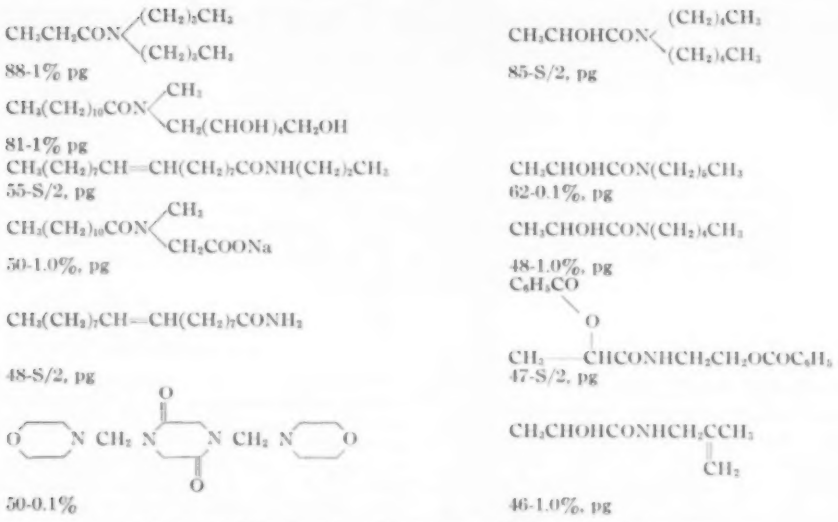

Fig. 2.—Inhibitory amides—More than 40% inhibition of glycolysis.

under test be able to diffuse through salivary centrifugate, to inactivate organisms or their enzyme systems that cause conversion of glucose to acid, and to have sufficient substantivity to withstand thirty-minute rinsing by the control buffer solution (12).

RESULTS

Of the total amides studied, there were 213 which contained no elements other than C, N, O, and H. Eight of these had residual inhibitory action of 50% or greater, and an additional four had inhibitory action between 40 to 49 per cent (Fig. 2). Below each structure the first figure represents the residual inhibitory action and the second is the concentration at test. The letters "pg" refer to a test in 10% propylene glycol and 90% water. For other tests, the solvent was water. The remainder of the amides tested were less inhibitory either because of unsuitable structure or poor solubility (Table I). Except for a piperazine derivative, the active substances were alkyl monoamides containing 7 to 21 carbon atoms. Three of the active chemicals were N,N-alkyl disubstituted propionamide or lactamide containing 11 and 13 carbon atoms. Apparently the range dibutyl to diamyl substitution affords optimum activity. Diethyl, di(2-hydroxyethyl), and di-2-hydroxypropyl derivatives of lactamide were inactive. The diallyl derivative is borderline, indicating that three carbon atoms are insufficient. The di-2-ethylhexyl and dioctyl derivatives are inactive, suggesting the activity falls off again with eight carbon atoms. Monosubstituted lactamides having methallyl, amyl, and hexyl substitution on the nitrogen were active. Tests have also been performed on lactamides with nitrogen substitution where the alkyl group had 2, 3, 4(butyl) 8, 10, 12, 14, 16, or 18 carbon atoms. None of these were active. The other inhibitory substances among amides were oleamide and its N-propyl derivative, but not its butyl derivative. N-Lauroyl sarcosinate was inhibitory but this is not a new compound, for its inhibitory action has been reported by Fosdick (8) who also reported it to be effective in a clinical trial (9). An N-substituted piperazine derivative was also inhibitory but this is not unusual since piperazine derivatives frequently have inhibitory action (11). There were no other inhibitory ring structures. There were seven acyclic acetamide derivatives, none of which were inhibitory. For the amide structures, it appears that N-substituted dibutyl, diamyl, or dihexyl propionamides or lactamides have inhibitory action, and that other alkyl amides may be active if they have more than 7 carbon atoms and their solubility in water or 10% propylene glycol is great enough.

Information was available on 71 urea derivatives, including some heterocycles involving urea. None of these had a residual influence when tested in aqueous solution. The compounds tried are pre-

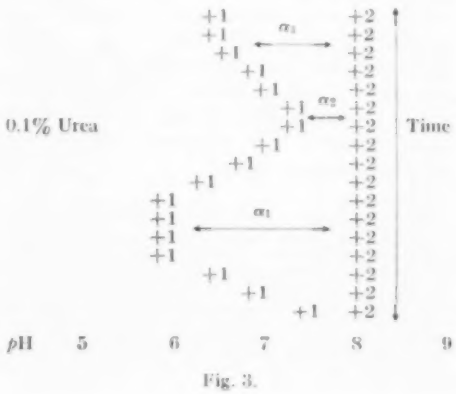

Fig. 3.

Inhibitory carbamates

87-S/2, pg

Inactive compounds with similar ring structures

1-Proline	1-Methyl-4-piperidone
1-Hydroxyproline	1-Acetyl piperidine
Nicotine (0.01%)	Two other piperidinyl compounds
N-Dodecyl pyrrolidine	

Inactive carbamates with five-membered heterocycles

57-S/2, pg

Carballyloxy oxazolidone	N-Methyl oxazolidone-2
Benzoxazolone	2-Hydroxy benzoxazole
4,4-Dimethyl-2-oxazolidone	3-Acetyl-2-oxazolidone
3-Butyl-2-oxazolidone	Four derivatives of 2,4-oxozolidinedione

Fig. 4.

sented in Table II. These include some which are common to Table I because they also possess an amide structure. The low incidence of activity makes it questionable as to whether urea derivatives deserve further study. Urea itself had no prolonged action, but during contact with dilute urea solutions the *p*H of the sediment is raised because of hydrolysis of urea by urease in the sediment (Fig. 3). Apparently the action of urease is highly specific. There were 50 derivatives of urea which were not hydrolyzed, including substances as similar to urea as biuret, hydantoic acid, 1,3-dimethyl and 1,3-diethyl, methyl, isopropyl, allyl and acetyl urea. The only substituted urea which caused a transient raising of *p*H of sediment was 1,1-diethyl urea.

The findings with 154 carbamates indicate a very low incidence of inhibitory action (Table III). Over 100 carbamates were tried before one was found with marked inhibitory action, and only two of the total appeared especially promising. These two were the N-carbo-isopropoxy derivatives of pyrrolidine and piperidine. The grouping appears to be highly specific. Five other pyrrolidine derivatives tested were negative, and three piperidine derivatives were without effect. There were 11 compounds having the entire carbamate structure in a five-membered heterocycle, and none of these exhibited inhibitory action.

TABLE I.—AMIDES HAVING LESS THAN 40% INHIBITORY ACTION

Inhibition 25–39%	
Acetyl *p*-aminobenzoic acid[b]	N-*n*-Hexadecyl lactamide[a]
p-Anisamide, N,N-diethyl-	Hydantoin[b]
4-(Benzylideneamino) antipyrine[a]	N-(2-Hydroxyethyl) lactamide dipropionate[a]
4,4′-Biantipyrine[b]	N,N-bis-(2-Hydroxyethyl) lactamide tripropionate
n-Butyl oxamate[b]	N-(2-Hydroxyethyl) ricinole amide[b]
N,N-Diallyl lactamide[a]	N,N′-bis(Hydroxy methyl) 2,5-diketo piperazine[b]
N,N-di-2-Ethylhexyl lactamide[a]	Lauric acid in propanol amide[a]
N,N-Dihexyl lactamide[a]	Phthalazone[c]
Di(*p*-Octyl benzyl dimethyl methyl amino ethanone) dichloride[c]	Piperidine, 1-*p*-anisoyl-
Gallic acid anilide[a]	2,5-Piperazinedione[b]
Inhibition 10–24%	
3-Acetyl aminobenzoic acid	Ethoxylated amide[a]
N-Acetyl-N-phenyl glycine (pract.)[a]	N-Ethyl lactamide[a]
Acetyl urea	Gallic acid amide[a]
Adipamide	Glycine anhydride[b]
Alloxantin[b]	Tetra-Glycyl glycine[a]
5-Amino-2,3-dihydro-1,4-phthalazine dione[a]	Hippuric acid[b]
2-Amino-4-phenyl-6-oxypyrimidine[a]	N-2-Hydroxyethyl lactamide[a]
p-Anisamide, N,N-diisopropyl-	N-(2-Hydroxyethyl) lactamide diacetate[a]
DL-Asparagine[a]	N-(2-Hydroxyethyl) palmitamide[a]
N-Benzyl lactamide[a]	N,β-Hydroxyethylphthalimide[b]
N-*n*-Butyl acetamide	N-tris (Hydroxymethyl) methyl lactamide[b]
N-Butylacetanilide[c]	2-Hydroxy-3-naphthoic acid hydrazide[b]
N-*n*-Butyl lactamide[a]	N-2-Hydroxy propyl lactamide
N-*n*-Butyl lactamide-2-ethyl hexanoate[a]	Lactanilide[a]
Caffeine citrate[b]	Maleic acid hydrazide[b]
3-Camphenilaneacetamide[a]	N-Methyl-lactanilide[a]
1-(Carboxy methyl)-pyridinum chloride hydrazide[b]	N-Methyloleamide[b]
Creatinine[a]	N-1-Methylpropyl lactamide[a]
N-Cyclohexyl lactamide (crude)[a]	N-2-Methylpropyl lactamide[a]
N-*n*-Decyllactamide[a]	3-Methyl-5-pyrazone[b]
N-*n*-Decyl nicotinamide[a]	Monomethylol dimethyl hydantoin[b]
Diacetoethylenediacetamide	5-Nitro-2-furaldehyde semioxamazone[a]
3,5-Diacetylaminophenol[a]	N-*n*-Octyllactamide[b]
N,N-Diallylmyristamide[a]	Orotic acid[b]

TABLE I.—*Continued.*—INHIBITION 10—24%

2,4-Diamino-6-oxypyrimidine[a]
Dibenzoyl-diamino-ethylene[b]
N,N-Dibutyl lactamide adipate[a]
N,N-Di-*n*-butyl lactamide acetate[a]
Diethyl acetamidomalonate[b]
N,N-Di-2-hydroxy-propyl lactamide[a]
N,N-Di-*n*-octyl lactamide[a]
5,5-Diphenyl-3-piperidylmethyl-2,4-[a]
N-*n*-Dodecyl lactamide[a]
N,N′-Bis(piperidyl methyl) 2,5-diketo-piperazine[b]
Pivalanilide[a]
N-*n*-Propyl lactamide (crude)[a]
5-Pyrimidinecarboxylic acid 1,2,3,4-tetrahydro-2,4-dioxo-, monohydrate
Salicylamide
Sodium glycocholate[b]
N-*n*-Tetradecyl lactamide[a]

Inhibition Less Than 10%

Acetamide[b]
Acetamide, N-sec-butyl-[c]
Acetamide, N,N-dibenzyl-
Acetamide, N-pentyl-[a]
Acetamide, N-propyl-[b]
Acetamidomalonic ester[b]
Acetanilide[b]
Acetanilide, N-isopentyl-
Acetanilide, N-pentyl-
Acetoacetanilide[b]
Acetphenetidin
Acetyl *p*-aminophenol[a]
Acetyl *p*-anisidine[b]
Acetyl dimethylamine[b]
Acetylglycine[b]
1-Acetyl-3-methylurea[b]
N-Acetylmorpholine[a]
N-Acetyloleamide[b]
3-Acetyl-2-oxazolidone
1-Acetyl piperidine
Allantoin[b]
Alloxan[b]
p-Aminoacetanilide[b]
4-Aminoantipyrene[b]
p-Aminobenzoyl glutamic acid[b]
p-Amino-stearanilide[b]
5-Aminouracil[b]
p-Anisamide, N,N-diethyl-[a]
Antipyrine[a]
Asparagine[b]
Azlactone of α-benzoylamino-β-(3,4-dimethoxy-phenyl) acrylic acid[b]
Barbituric acid[b]
N-Benzoyl-β-3-4, dimethoxyphenyl (*dl*) α-alanine[b]
Brucine[b]
Butyramide, N-benzyl-
p-Butyrotoluidide[a]
Caffeine[b]
Cinnamamide, N,N-dibutyl-[c]
Creatinine, 5-benzylidene-
Diacetoethylenediacetamide[a]
3,5-Diacetylaminoanisole[a]
1,3-Di(4-antipyrinyl) urea[a]
N-N-Dibenzyl lactamide acetate[a]
N-N-Dibutyl lactamide laurate[a]
3-β-Diethylaminoethyl-5,5-diphenyl-2,4-oxazolidine-dione
3-β-Diethylaminopropyl-5,5-diphenyl-2,4-oxazoli-dinedione
Diethylformamide
N,N-Diethyl lactamide[a]
N,N-Di(2-hydroxyethyl) lactamide[a]
2,5-Diketo-6-carbethoxy piperazine[b]
2,3-Diketo piperazine[b]
N,N-Dimethylformamide[b]
Dimethyl hydantoin[b]
Dimethyl hydantoin-formaldehyde resin[b]
N-1,1-Dimethyl-3-hydroxybutyl lactamide[b]
3,6-Dimorphylethyl-2,5-diketopiperazine[b]
Diphenylacetamide[a]
5,5-Diphenylhydantoin[b]
1,3-Diphenyl-5-pyrazolone[a]
N-Ethyloleamide[c]
Ethyl oxanilate[a]
Formamide[b]
Furoamide
Furylacrylamide[b]
Glucuronamide
L-Glutamine[b]
Glycine anhydride[b]
Glycyl glycine HCl[b]
Hydrogenated tallow amide[a]
N-2-Hydroxy ethyl glycolamide[c]
N-N-Bis (2-hydroxyethyl) lactamide triheptanoate[a]
N-(2-Hydroxy ethyl) lauramide[b]
N-(2-Hydroxyethyl) ricinole amide[c]
4-Imidazolidinone, 3-(hydroxymethyl)-2-(hydroxy-methylimino)-1-methyl-
Isatin[b]
Lactamide acetate[b]
Malonamide[b]
Methacrylamide[b]
N,N′-Methylenebis-acrylamide[b]
N-1-Methyl ethyl lactamide (N-isopropyl lactamide)
N-2-Methyl-2-hydroxy propyl lactamide[b]
5-Methylisatin[b]
3-Methyl-1-phenyl-5-pyrazolone[b]
1-Methyl-2-pyridone[b]
Morpholine, 4-*p*-anisoyl-
3-β-Morpholinoethyl-5,5-diphenyl-2,4-oxazolidine-dione[a]
Murexide[b]
Myristamide[c]
N-(5-Nitro-2-furfurylidene)-1-amino hydantoin[a]
N-*n*-Octadecyl lactamide[c]
N-Octadecyl nicotinamide chloride[b]
Oxamide[b]
Palmitamide[c]
Pantothenol[b]
α-Phenoxyacetamide[a]
Phenylacetamide[b]
2-Phenyl benzoxazole[b]
2-Phenyl, 4-(3,4-dimethoxybenzal) oxazolen-2-one-5[b]
1-Phenyl-3-methyl-5-pyrazolone
Piperidine, 1-acetyl-[b]
Polyvinyl pyrrolidine[a]
Procaine amide HCl[b]
Propionanilide, N-butyl-
Propionanilide, N-ethyl-
Propionanilide, N-isopentyl-
Propionanilide, N-pentyl
N-(*n*-Propyl) oleamide[c]
Riboflavin[b]
Serine anhydride 2,5-dihydroxymethyl-3,6-diketo-piperazine[b]
Sodium Nembutal[b]
Sodium urate[b]
Sodium violurate
Succinimide[b]
N,N,N′,N′-Tetraethylphthalamide
Uric acid[b]
Uracil[b]
Uramil[b]
Xanthine

[a] pg 1.0% or 0.5 ss; [b] H_2O 0.1 ss or less; [c] pg 0.1 ss or less. Unmarked compounds, H_2O 1.0% or 0.5 ss.

TABLE II.—UREA DERIVATIVES

Inhibition more than 40%

Isopropylurea[a]
1,3-Diethylurea[a]

Inhibition 15–39%

Alloxantin[b]
Carbohydrazide, 1-carbamoyl-[a]
1-Diethylcarbamyl piperazine[b]
1-Diethyl carbamyl-2,4,5-trimethyl piperazine[b]
Hydantoin[b]
Guanylurea phosphate[b]
Guanylurea sulfate[b]
5-Nitro-2-furaldehyde-2-methyl semicarbazone[a]
Phenylurea[a]
Urea oxalate[a]

Inhibition less than 15%

Acetylene diureine[b]
1-Acetyl-3-methylurea[b]
Acetyl urea[b]
Allantoin[b]
Alloxan[b]
2-Allyloxy-4,6-diamino-1,3,5-triazine[b]
Allylurea
5-Aminouracil[b]
Ammelide[b]
Ammeline[b]
Barbituric acid[b]
Biuret[b]
Biurea[b]
Tert-butylurea[b]
Caffeine[b]
Caffeine citrate[b]
DL-Citrulline[b]
Cyanuric acid[b]
1,3-Di(4-antipyrinyl) urea[a]
1-Diethyl carbamyl-4-amino piperazine[b]
1-Diethylcarbamyl-4-(*p*-dimethylaminobenzal-amino) piperazine[b]
1-4-Bis(diethyl carbamyl) 2,5-dimethyl piperazine[b]
1,1-Diethylurea
1,3-Diethylurea[a]
1,5-Dioctadecyl-biuret[b]
Dimethyl hydantoin[b]
Dimethyl hydantoin-formaldehyde resin[b]
1,3-Dimethylurea[b]
Di-*p*-phenetylurea[b]
Diphenylcarbazide[b]
Diphenylurea[b]
Di-α-pyridylurea[b]
Dodecylurea[b]
Dodecylurea N-glucoside[b]
p-Ethoxyphenylurea (Dulcin)
Furacin[b]
Furfural semicarbazone[b]
Hydantoic acid[b]
Isopropylurea[a]
Methylurea[b]
Monomethylol dimethyl hydantoin[b]
5-Nitrobarbituric acid[b]
Orotic acid[b]
p-Phenetylurea[b]
1-Phenyl-3-(4-benzyl-1-piperazyl) urea[b]
Phenyl-3-morpholinylpropylurea[a]
4-Phenyl semicarbazide[b]
5-Pyrimidinecarboxylic acid, 1,2,3,4-tetrahydro-2,4 dioxo-, monohydrate
Semicarbazide hydrochloride[b]
Sodium Nembutal[b]
Sodium urate[b]
Sodium violurate
Tetrabutylurea[a]
m-Tolylurea[b]
Triallyl cyanurate[b]
Uric acid[b]
Uracil[b]
Uramil[b]
Urea
Urea, 1H-tetrazol-5-yl-

Footnotes: See Table I.

TABLE III.—CARBAMATES HAVING LESS THAN 40% INHIBITION

Inhibition 25–39%

Benzoxazolone[a]
n-Butyl carbamate[a]
Carballyloxy oxazolidone[a]
Carbamic acid, isopentyl-, 2-hydroxy-ethyl ester[a]
α-Carbobutoxyethyl N-3-methylphenylcarbamate[a]
α-Carbobutoxyethyl N²-phenylcarbamate[a]
β-Cyanoethyl N-phenylcarbamate[a]
1-(1-Cyano-3,5,5-trimethyl) hexyl N-phenylcarbamate[a]
α,β-Dicarbobutoxyethyl N-phenylcarbamate[a]
Diethylene glycol bis-carbamate[a]
2,5-Dimethyl-2-5-dicarbanilino-3-hexyne[a]
3,7-Dimethyl-1,6-octadien-3-yl N-phenylcarbamate[a]
4,4′-Diphenylene bis-(*o*-isopropylcarbamate)[a]
Ethyl N-methyl carbamate[a]
5-Indanyl N-3-chlorophenyl carbamate[a]
N,N[bis-Isoamyl] β-hydroxyethyl carbamate[a]
Isopropyl N-2,4-dimethoxyphenylcarbamate[a]
Isopropyl N-2,5-dimethoxyphenylcarbamate[a]
Isopropyl N-2,4-dimethylphenylcarbamate
Isopropyl N-2,5-dimethylphenylcarbamate[a]
Isopropyl N-3-ethoxyphenylcarbamate[a]
Isopropyl N-ethyl-N-phenylcarbamate[a]
Isopropyl N-2-furfurylcarbamate[a]
Isopropyl N-2-methoxy-5-methylphenylcarbamate[a]
Isopropyl N-3-methoxyphenyl carbamate[a]
Isopropyl N-methyl-N-3-methylphenylcarbamate[a]
Isopropyl N-methyl-N-4-methylphenylcarbamate[a]
Isopropyl N-methyl-N-phenylcarbamate[a]
Isopropyl N-3-methylphenylcarbamate[a]
Isopropyl N-3-(morpholinyl)-*n*-propyl carbamate[a]
Isopropyl N-3-vinylphenylcarbamate[a]
Lactic acid, carbanilate, cyclohexyl ester[a]
α-Methyl-α-carboethoxyethyl N-phenylcarbamate[a]
β-(N′-Morpholinyl) ethyl N-phenylcarbamate[a]
m-N,N′-Phenylene bis-(allyl carbamate)[a]
m-Phenylene bis-(o-isopropylcarbamate)[a]
β-(2-Pyridyl) ethyl N-phenyl carbamate[a]
3-Pyridyl N-phenyl carbamate[a]
DL-Trans-3,3,5-trimethyl cyclohexyl N-phenyl carbamate[a]
8-Quinolyl N-phenyl carbamate[a]

Inhibition 10–24%

Acetoxime N²-phenylcarbamate[a]
Carbamic acid, dodecyl-, 2-hydroxyethyl ester[a]
Ethylene bicarbamate[a]
2-(2-Ethynyl) butyl N-phenylcarbamate[a]

TABLE III.—*Continued.*—INHIBITION 10–24%

Carbamic acid, ethylenedi-, bis (2-hydroxyethyl) ester
1-Carbethoxy-4-amino piperazine hydrochloride[a]
N-Carbethoxy piperazine
α-Carbobutoxybenzyl N-phenylcarbamate[a]
α-Carbobutoxyethyl N-3-cyanophenylcarbamate[a]
α-Carbobenzoxyethylcarbamate[a]
α-Carbobutoxyethyl N-ethylcarbamate[a]
α-Carbobutoxyethyl N-2-methoxy-5-methyl-phenylcarbamate[a]
α-Carbobutoxyethyl N-methyl-N-phenyl-carbamate[a]
α-Carbobutoxyethyl N-phenylcarbamate[a]
α-Carboethoxyethyl N-phenylcarbamate[a]
α-Carboxyethyl N-phenylcarbamate[a]
Cyclopentyl N-phenylcarbamate[a]
N-Decyl β-hydroxy ethyl carbamate[a]
Diethylene glycol bis(N-di-)(2-hydroxyethyl) carbamate
Diethylene glycol bis-(N-2-hydroxyethyl) carbamate[a]
3,3′-Dimethoxy-4,4′-diphenylene bis (*o*-isopropyl carbamate)[a]
3,7-Dimethyl-2,6-octadien-1-yl-N-phenylcarbamate[a]
3,7-Dimethyl-6-octen-1-yl-N-phenylcarbamate[a]
1,1-Dimethyl-2-propynyl N-phenylcarbamate
3-Formylphenyl N-phenylcarbamate[a]
Furfuryl N-phenylcarbamate[a]
β-1(2-Heptadecenyl)glyoxalidinylethyl N-phenyl-carbamate[a]
Isopropyl N-benzyl-N-phenylcarbamate[a]
Isopropyl N-3-cyanophenylcarbamate[a]
Isopropyl N-3,5-dimethylphenylcarbamate[a]
Isopropyl N-2,6-dimethylphenylcarbamate[a]
Isopropyl N-4-ethoxyphenylcarbamate[a]
Isopropyl N-2-methyl-5-isopropylphenylcarbamate[a]
Isopropyl N^2-Methyl-N^2-phenyl carbamate
Isopropyl N-2-(5-methyl) pyridylcarbamate[a]
Isopropyl N-2-(6-methyl) pyridylcarbamate
Isopropyl N-phenyl carbamate[b]
Isopropyl N-(2-pyrimidyl)carbamate[b]
2-Methylallyl N-phenylcarbamate[a]
α-Methyl-α-cyanoethyl N-phenylcarbamate[a]
4-Morpholine ethanol, carbanilate (ester)[a]
N-Octadecyl β-hydroxy ethyl carbamate[a]
β-(1-Phenoxy)propyl N-phenylcarbamate[a]
Propynyl N-phenylcarbamate[a]
N-"RDsoya" β-hydroxy ethyl carbamate[a]
(2-Tetrahydropyranyl)-methyl N-phenyl carbamate[a]
dl-Cis-3,3,5-trimethylcyclohexyl N-phenyl-carbamate[a]

Inhibition Less than 10%

1-Allyl-4-carbethoxy piperazine[b]
Benzaldehyde, *m*-hydroxy-, carbanilate[a]
N-Benzyl β-hydroxy ethyl carbamate[a]
Butyl carbamate[b]
3-Butyl-2-oxazolidone[a]
Carbamic acid, diphenyl-, ethyl ester[b]
Carbamic acid, dodecyl-, 1(or 2)-mono-ester with 1,2-propanediol[a]
Carbamic acid, (2-hydroxyethyl)-, 2-hydroxyethyl ester
Carbamic acid, bis(2-hydroxyethyl)-, 2-hydroxyethyl ester
Carbamic acid, 2-hydroxypropyl ester
Carbanilic acid, N,*o*-dimethyl-, isopropyl ester[c]
Carbanilic acid, N,*p*-dimethyl-, isopropyl ester[a]
1-Carbethoxy-4-*n*-heptyl piperazine[b]
1-Carbethoxy-4-methyl piperazine oxide monohydrate[b]
α-Carbocyclohexoxyethyl N-phenylcarbamate[a]
α-Carbododecoxyethyl N-phenylcarbamate[a]
N-decyl β-hydroxy propyl carbamate[a]
N,N′-Dicarbo-ethoxy hexylene diamine
Diethylene glycol dicarbamate[b]
2-Dimethylamino ethyl-1-piperazine carboxylate[b]
4,4-Dimethyl-2-oxazolidone
3,4-Dimethyl-5-phenyl-2-oxazolidone[a]
N-Dodecyl β-hydroxyethyl carbamate[a]
N-Dodecyl β-hydroxy propyl carbamate[a]
Dodecyl urethane[b]
Ethyl carbamate
Ethyl-N-(4-carbethoxy-1-piperazyl) carbamate[b]
Ethyl N,N-diphenylcarbamate[a]
Ethyl-N-ethylcarbamate[b]
Ethyl N-ethyl-N-phenylcarbamate[a]
N-Hexadecyl β-hydroxy ethyl carbamate[a]
N-Hexadecyl β-hydroxy propyl carbamate[a]
2-Hydroxybenzoxazole[b]
β-Hydroxy ethyl carbamate
N,N[Bis-β-hydroxy ethyl] β-hydroxy ethyl carbamate
β-Hydroxy propyl carbamate
Isopropyl N-*n*-butyl-N-phenylcarbamate[a]
Isopropyl N-cyclohexylcarbamate[b]
Isopropyl N-2,5-diethoxyphenyl carbamate[a]
Isopropyl N-dehydroabietylcarbamate[a]
N-Isoamyl β-hydroxyethyl carbamate[a]
Isopropyl N-isoamyl-N-phenyl carbamate[a]
Isopropyl N-methyl-N-2-methylphenylcarbamate[a]
Isopropyl N-(2-pyridyl)carbamate[b]
N-Methyl β-hydroxy ethyl carbamate
N-Methyl oxazolidone-2
N-Octadecyl β-hydroxy propyl carbamate[a]
β-Phenylethyl N-phenylcarbamate[a]
1,4-Piperazinedicarboxylic acid, 2,5-dimethyl-, diisopropyl ester[a]
n-Propyl carbamate[b]
2-Pyridinecarbamic acid, 4,6-dimethyl-, isopropyl ester[a]
N-"GD Tallow" β-hydroxy ethyl carbamate[a]
N-Tetradecyl β-hydroxy ethyl carbamate[a]
N-Tetradecyl β-hydroxy propyl carbamate[a]
1H-Tetrazole-5-carbamic acid, ethyl ester[a]
Urethane (ethyl carbamate)[b]

Footnotes: See Table I.

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salicylate, theophylline, sulfanila-

Illustrations and Pharmaceutical Interpretations of First Order Drug Elimination Rate from the Bloodstream*

By JOSEPH V. SWINTOSKY

Literature data have been employed to illustrate approximate first order drug elimination from the bloodstream. Data are presented for sulfathiazole, sulfapyridine, theophylline, salicylate, penicillin, and tetracycline. Some interpretations of the possible significance of such drug elimination rate to dosage regimens and dosage form design are discussed.

FOR MOST DRUGS, graphic, mathematic, or other convenient methods have not yet been developed to delineate accurately therapeutic response as a function of time. However, for some drugs a satisfactory therapeutic response may be associated with a blood level range; and where drug blood levels may be determined with a measure of accuracy, one may employ Cartesian coordinate plots to describe drug blood levels as a function of time. Such a curve may be used as a qualitative guide to dosage form design and dosage regimens. The slope of the curve may be approximated at any time, and is a measure of the rate of change of drug concentration in the blood.

A more elegant and useful analysis of blood level data appears applicable when a specific velocity constant or half-life constant can be used to describe the drug elimination rate at specific dosage levels or drug tissue levels. When single drug blood levels or urinary excretion rates are analyzed under conditions of initial maximum dosage for prolonged time intervals, it is commonly observed that mathematical expressions defining the excretions and biotransformations for the elimination processes become complex because of the superimposition of several rate terms. These elimination rates in humans may be dependent upon the dose, mode of administration, tissue levels, distribution in the tissues, storage or fixation in the tissues, kidney threshold, state of health, acid-base balance, individual idiosyncrasies, etc. Lapp (1), in describing his investigations and those of other foreign investigators in interpreting urinary drug excretion rates, has illustrated for some drugs the resolution of the combined rate terms as they govern the shape of the excretion rate curve for a given drug.

EXPERIMENTAL

Significance of First Order Rates.—By confining the analysis to restricted blood level ranges, some useful information on the kinetics of drug elimination may be derived from blood level data. For some drugs the therapeutic response appears to be related to the drug blood levels. When one predominant mechanism for drug elimination exists in a useful therapeutic blood level range, the description of the elimination rate might be approximated by a simple rate expression. It is the purpose of this paper to illustrate this with data from the literature which can be described by first order rates. Also presented are some interpretations of the possible significance of such excretion, biotransformation or other methods of first order drug disappearance to absorption rate, dosage form design and dosage regimens.

Determination of First Order Elimination Rates.—First order drug elimination rate from the bloodstream is defined by the equation: $\log C = -(k_b/2.303)\,t + \log C_o$, where C = drug concentration in the blood; k_b = a biologic velocity constant characterizing drug elimination from the bloodstream; t = time after drug administration; and $\log C_o$ = a constant corresponding in magnitude to the intercept on the concentration axis at zero time when $\log C$ is plotted vs. t for the descending blood level curve. Similarly the biologic half-life of the drug in the bloodstream may be represented as: $(t_{1/2})_b = 0.693/k_b$, where $(t_{1/2})_b$ signifies the time required for a given blood drug concentration to fall to one half this value in a blood level range where first-order elimination rate holds. The constants with subscripts are recommended to indicate that they are derived from blood level data and refer to drug half-life and elimination rate observed for intravenously administered doses at approximately therapeutic levels, unless specified otherwise.

When drug elimination rate approaches first order, $\log C$ vs. t approximates a straight line. Axelrod and Reichenthal (2) have demonstrated such elimination rates for orally and intravenously administered caffeine. In different human subjects the $(t_{1/2})_b$ ranged from two and one-half to four and one-half hours. Lous (3) has observed approximately straight line elimination of barbital, phenobarbital, and aprobarbital (N.N.R.) from the bloodstream when plotted in this manner. Dominguez and Pomerene (4) describe this type of elimination for creatinine and other nondrug substances; and Boxer, *et al.* (11), for streptomycin. A cursory examination of the scientific literature reveals that a similar relationship appears to exist for a number of common drugs such as penicillin, tetracycline,

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TABLE I.—HUMAN BLOOD LEVEL DATA FOR SEVERAL DRUGS AS DESCRIBED IN THE LITERATURE

Drug	Reference	Human Subjects	Route*	Dose, Gm.	Drug measured in Bloodstream	Blood Level of Free Drug, mg./100 cc. Hours Following Administration 1	2	3	4	5	6	7	8	9	10	12	16	24
Sulfathiazole	(5)	Case No. 37	I	1.841	Sulfathiazole	4.7	..	3.3	..	2.3	..	1.9	..	..	..	..	..	..
		Case No. 38	I	1.841	Sulfathiazole	5.0	4.0	..	3.2	..	1.8	..	..	..	..	..	..	..
		Case No. 39	I	1.841	Sulfathiazole	5.2	4.1	..	2.9	..	2.0	..	..	..	..	..	..	..
		Case No. 40	I	1.841	Sulfathiazole	5.2	3.9	..	2.6	..	1.8	..	..	..	..	..	..	..
Sulfapyridine	(5)	Case No. 7	0	4	Sulfapyridine	2.3	2.7	..	3.6	..	3.0	..	..	..	2.0	..	..	..
		Case No. 18	0	4	Sulfapyridine	1.8	2.8	..	3.9	..	3.5	..	2.6	..	2.2	..	..	..
		Case No. 37	I	1.841	Sulfapyridine	3.3	..	2.8	..	2.2	..	1.8	..	..	..	..	..	..
		Case No. 39	I	1.841	Sulfapyridine	3.8	3.4	..	2.6	..	2.1	..	..	..	..	..	..	..
		Case No. 40	I	1.841	Sulfapyridine	3.7	3.3	..	2.7	..	2.3	..	..	..	..	..	..	..
Aminophylline	(6)	Av., 11 Subjects Corr. to Body Wt. of 70 Kg.	I	0.5	Theophylline	0.9	0.74	0.64	0.55	0.44	0.31	0.27	0.21	..	..	..	..	..
		1 Subject Body Wt. Corr. to 70 Kg.	0	0.5	Theophylline	0.22	0.47	..	0.63	..	0.50	..	0.29	..	..	..	..	..
Sodium salicylate	(7)	Av., Cases Nos. 1–4; 6–7; 9–11.	I	10	Salicylic acid	39.4	..	..	..	..	31.4	..	..	..	..	24.2	..	16.2
		Av., Cases Nos. 1–4.	0	10	Salicylic acid	5.0	..	..	..	..	14.4	..	..	..	..	15.7	..	12.5
		Av., Cases Nos. 1, 2, and 6	I	20	Salicylic acid	56.7	..	..	..	..	43.0	..	..	..	..	35.2	..	26.6
Tetracycline hydrochloride	(10)	Av., 6 subjects	I	0.5	Tetracycline hydrochloride	..	..	..	0.48	..	..	..	0.31	..	..	0.24	0.15	..

* I = Intravenous administration; 0 = oral administration.

salicylate, theophylline, sulfanilamide, and some derivatives related to the latter compound. Some of these are illustrated in the data of Table I and Fig. 1–6.

From the various intravenous data in the figures it is obvious that approximately straight line relationships exist in the blood level ranges depicted. The $(t_{1/2})_b$ can be readily determined either analytically or graphically and approximate values for $(t_{1/2})_b$ and k_b, as determined from these data, are tabulated in Table II. The ability to describe drug blood levels and elimination rates in terms of biologic constants is not only of practical importance but of considerable convenience because they are scalar quantities which lend themselves to comparison and tabulation. In Table II, there is considerable difference in the magnitudes of $(t_{1/2})_b$ values as represented for these few drugs, with the value for penicillin relatively small and for salicylate relatively large.

Sulfathiazole and Sulfapyridine.—A rather remarkable uniformity in free sulfathiazole elimination rate, in four different subjects, is demonstrated in Fig. 1 which shows blood levels observed in patients receiving 1.841 Gm. intravenous doses of sulfathiazole. Without regard for possible variations in body weight, the combined data plot satisfactorily as a single straight line. For these data k_b = 17%/hour and C_o = 5.8.

Figure 2 shows free sulfapyridine blood levels in five human patients of which three received single 1.841-Gm. intravenous doses and two received 4-Gm. oral doses. The $(t_{1/2})_b$ for the intravenously administered drug varied between five and seven hours. This and other data (1, 2) suggests that even in a restricted blood level range, the $(t_{1/2})_b$ is not a fixed absolute value, but like other biologic parameters is probably subject to variations effected by physiologic differences in age and sex, states of health, diet patterns, specific idiosyncrasies, etc. Thus it appears that $(t_{1/2})_b$ must be considered a statistical average value for a population, subject to variations, the degree of which is probably also related to the drug in question. Hence k_b and $(t_{1/2})_b$ are best represented as average values and ranges of normal values at specified dosage levels; and their accurate tabulations depend upon a considerable number of experimental determinations. The data of Table II therefore should be tempered in the light of these remarks.

essary to give oral penicillin very frequently, or to

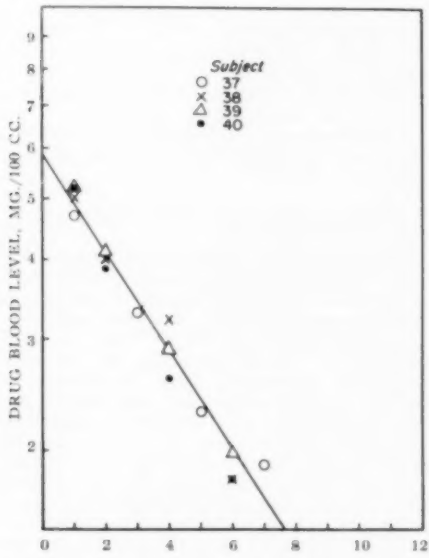

Fig. 1.—Sulfathiazole blood levels for four human subjects receiving 1.841-Gm. intravenous doses. Data from reference (5).

It is of interest to note that four hours after administration, the slopes of the orally-administered sulfapyridine are similar to those of the drug given intravenously. This suggests that effective absorption into the bloodstream of the 4-Gm. oral doses of sulfapyridine in these patients ceased after four hours. Thus this method of plotting data for drugs, exhibiting approximate first order elimination rate, might permit a close estimation of the rate and period of drug absorption into the bloodstream (4). One must assume, however, that the drug enters the bloodstream directly without first being sequestered selectively and in appreciable amounts in any tissue or organ for very extended periods.

Theophylline.—Figure 3 illustrates the average theophylline blood levels of eleven human subjects (corrected to body weights of 70 Kg.) receiving 0.5 Gm. aminophylline intravenously, and one subject receiving the same dose orally. For the oral dose, effective absorption into the bloodstream appears to have ceased after four to five hours when the slopes of the oral and intravenously-administered drug become essentially equal. Also, qualitative inspection of these plots suggests rather complete absorption of the drug into the bloodstream from the gastrointestinal tract.

TABLE II.—APPROXIMATE BIOLOGIC HALF-LIFE, $(t_{1/2})_b$, AND VELOCITY CONSTANT, k_b, FOR BLOOD STREAM ELIMINATION RATE OF SEVERAL DRUGS ADMINISTERED INTRAVENOUSLY

Drug	$(t_{1/2})_b$, hr.	k_b (0.693/$(t_{1/2})_b$)	Approximate Blood Level Range Studied, mg./100 cc.
Sulfathiazole	4	0.17	1.5–6
Sulfapyridine	6.5	0.11	1.5–4.5
Theophylline	3	0.23	0.2–1.0
Salicylic acid	19	0.036	10–60
Penicillin G	<1	<0.7	0.01–0.50 (Florey u./cc.)
Tetracycline	8	0.087	0.15–0.5

Salicylate.—Salicylate elimination rate at 10 and 20 Gm. intravenous dose levels of sodium salicylate is illustrated in Fig. 4. Although the elimination rates for a group of subjects at each dosage level correspond rather closely, it is interesting to note that the zero time blood levels do not vary by a factor of 2. Although one might speculate that greater tissue fixation or storage might occur at the higher levels, also accounting for the somewhat larger $(t_{1/2})_b$ observed at the higher blood levels, the reason for the considerable divergence is not clear; however, this phenomenon also occurs quite commonly with other drugs. From the shape of the curve derived from the subject receiving the single oral 10-Gm. dose, one might conclude that at this dose level salicylate is absorbed over a very protracted time interval (twelve hours or more). Moreover, oral absorption into the bloodstream at this dosage level did not appear to be complete even after twenty-four hours, otherwise, the 10-Gm. oral and intravenous dose curves should have intersected, assuming, of course, that the weight of the subject receiving the oral dose was near the average weight of the subjects receiving the 10-Gm. intravenous doses; and that elimination rate for this individual was typical of the larger group.

The large $(t_{1/2})_b$ of salicylate has special implications. It would appear in some instances that the cumulative effects of certain drugs might be related to this value; and that salicylism may be a direct result of an overly ambitious dosage regimen prescribed without regard for the approximately first order elimination rate and the relatively large

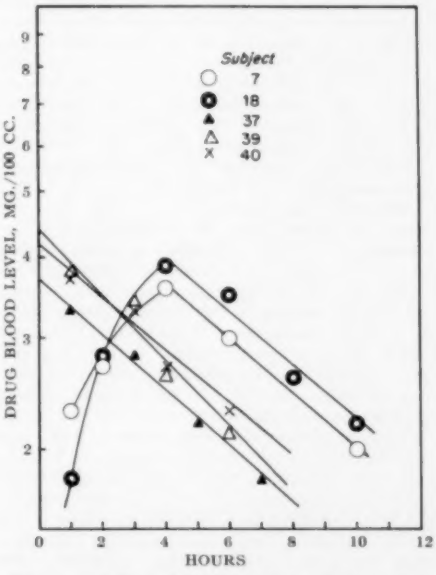

Fig. 2.—Sulfapyridine blood levels for three human subjects receiving 1.841-Gm. doses of intravenous drug, and two subjects receiving 4-Gm. oral doses. Data from reference (5).

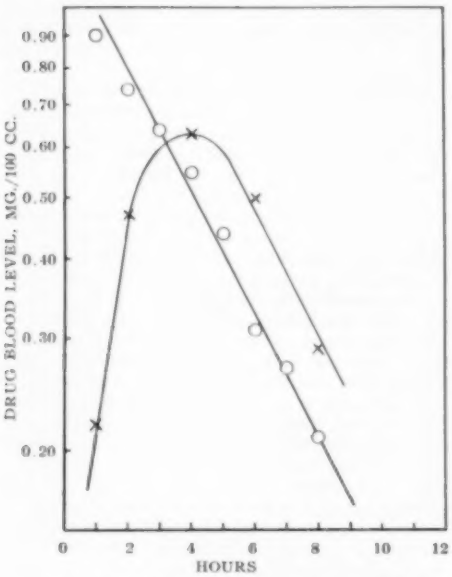

Fig. 3.—Average theophylline blood levels for 11 human subjects receiving 0.5 Gm. intravenous aminophylline, and 1 subject receiving 0.5 Gm. aminophylline orally. Data from reference (6).

$(t_{1/2})_b$ of the salicylate. For a drug of this type a relatively large dose may be required to yield a therapeutic blood level, yet once established only relatively small, infrequent sustaining doses would be necessary to maintain the initial level. Herein appears to lie an advantage of knowing the $(t_{1/2})_b$ of a compound. When $(t_{1/2})_b$ or k_b can be determined by intravenous or other routes of administration, and a correlation between blood and therapeutic levels exists, the data so obtained should enable one to calculate the magnitude and frequencies of doses required to attain and sustain the approximate therapeutic drug level desired.

Penicillin.—Rammelkamp, *et al.* (8), have obtained blood level data for a 10,000-Florey Unit intravenous dose of penicillin G sodium administered to three human subjects. The average blood levels observed at intervals up to ninety minutes, recorded graphically in Fig. 5, reveal that penicillin in these concentrations is eliminated extremely rapidly from the bloodstream, approaching first order elimination. On the other hand, when Foltz and Schimmel (9) (Fig. 6) administered 300,000 units of oral penicillin G potassium to twelve human subjects they observed blood levels of 1.28, 1.19, 0.55 and 0.177 U. S. P. U./cc. at $^1/_2$, 1, 2, and 4 hours, respectively, a rate of decrease in penicillin blood level considerably slower than that observed for the intravenously administered drug. This curve suggests a very rapid penicillin absorption from the gastrointestinal tract. However, after about $^1/_2$ hour, the amount of penicillin absorbed into the bloodstream decreases but may be sufficient to sustain blood levels better than is observed for intravenous doses. This suggests why it is necessary to give oral penicillin very frequently, or to use continuous drip methods in intravenous administration of penicillin if relatively uniform blood levels are to be maintained.

Tetracycline.—Figure 6 also describes blood levels observed for intravenously administered tetracycline hydrochloride. A considerable difference in the $(t_{1/2})_b$ of this antibiotic substance, and penicillin, is observed.

APPLICATION OF THESE FINDINGS

Although the data presented here for individual drugs are somewhat limited, compositely they show, in a restricted blood level range, an approximate first order elimination rate. When first-order elimination rates apply to any class of drugs, they lend themselves to valuable deductions and theoretical calculations particularly when therapeutic response may be related to the drug blood levels. Theoretically a few drug blood levels determined through the desired range for an intravenously administered dose are all that are necessary to interpolate the continuous blood level curve. When the straight line relationship is established on semilog graph paper, the shape of the drug blood level curve on Cartesian coordinate paper is readily determined. The k_b and $(t_{1/2})_b$ values become simple and useful biologic constants by which drug elimination rates may be characterized. The use of these constants can be helpful in a calculation of the theoretical magnitudes and frequencies of drug doses to be administered in order to attain and maintain the blood levels desired.

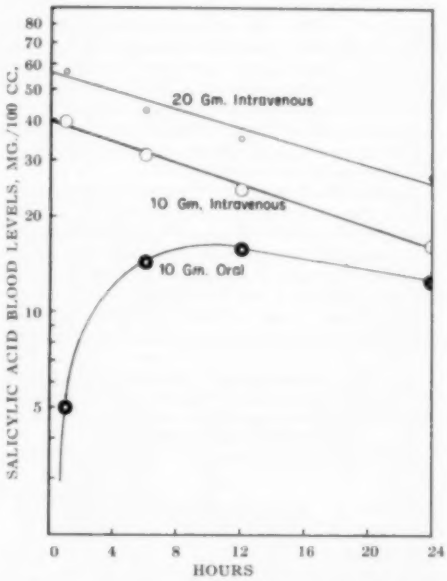

Fig. 4.—Average salicylic acid blood levels for 9 human subjects receiving 10 Gm. intravenous, 3 subjects receiving 20 Gm. intravenous, and 4 subjects receiving 10 Gm. oral, sodium salicylate. Data from reference (7).

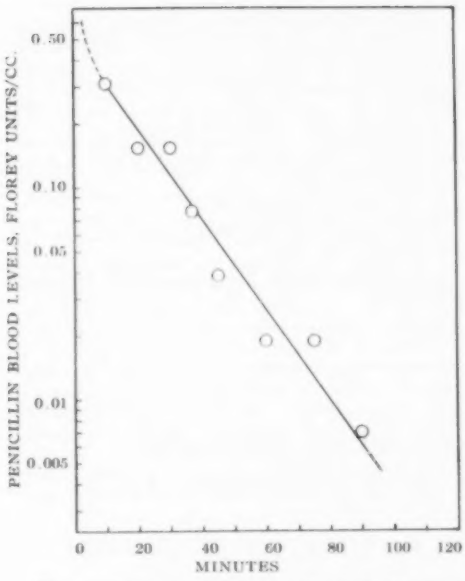

Fig. 5.—Average penicillin blood levels for 3 human subjects receiving 10,000 Florey Units of penicillin G sodium intravenously. Data from reference (8).

Similar rationalization may apply to an orally administered drug when first order elimination rate exists. Sufficient blood level data must first be obtained to delineate the blood level curve during the absorption phase. Thereafter when absorption ceases and the rate of drug disappearance approximates that for intravenously administered drug, then minimal experimental data would be required to interpolate the continuous blood level curve. For the latter type curve, at least for some drugs, it should be possible to determine: (*a*) an oral absorption rate curve; (*b*) an excretion rate curve; (*c*) the time at which absorption and excretion rates are equal; (*d*) the time at which absorption ceases; (*e*) the approximate percentage of the dose absorbed and excreted, and other useful information. Such data should also permit reasonable estimates of the useful dose to be incorporated into a given oral dosage form, the design of this dosage form and the frequency of administration in order to attain and maintain desired blood levels.

Theoretically, when intramuscular, subcutaneous, sublingual, rectal or other routes of administration are employed with drugs exhibiting first order elimination rates by the intravenous route, semilog graph paper plots afford a logical and convenient basis for evaluation of the rates, times, and efficiencies of absorption, release, action, and elimination. This approach constitutes a more convenient refinement of methods now employed to guide the pharmacist in dosage form design and to aid the medical investigator in evaluating the effect of various dosage forms, dosage regimens, and other variables affecting the blood levels of drugs and related therapeutic response.

SUMMARY AND CONCLUSIONS

1. Literature data have been presented to illustrate the approximate first order elimination rate from the human blood stream of sulfathiazole, sulfapyridine, theophylline, salicylate, penicillin, and tetracycline within therapeutic ranges. For this type of drug elimination, the biologic half-life and velocity constant for drug elimination rate, through a given blood level range, are readily determined. For the intravenously administered drugs, at the blood levels studied, the average half-life of each drug varied from a minimum value less than one hour for penicillin G sodium to approximately nineteen hours for salicylate. This first order elimination rate at therapeutic drug levels is demonstrable for numerous other drugs.

2. The concept as described for intravenously administered drugs also has application to orally administered drugs. For some of these, semilog paper plots of the blood level curves employed in conjunction with appropriate calculations should permit determination of curves of absorption and excretion rates and other useful information. Such data should have application to the design of oral dosage forms and regimens. Similarly the concept may be applied to the design and

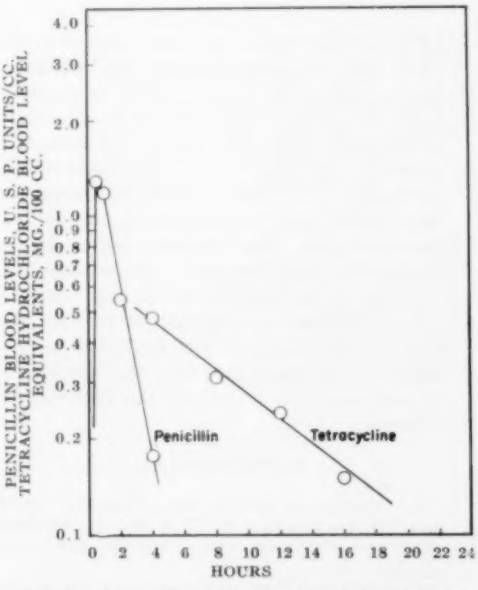

Fig. 6.—Average penicillin blood levels for 12 human subjects receiving 300,000 U. S. P. Units oral penicillin G potassium; and average blood levels for 6 subjects receiving 0.5 Gm. intravenous tetracycline hydrochloride. Data from references (9, 10).

evaluation of other dosage forms and routes of administration.

3. For drugs not exhibiting this type of elimination rate, other mathematical or graphical expressions may be derived to define the elimination process in terms of useful biologic parameters. When this is possible by means of drug blood levels or other experimental approach, the information should be especially valuable in the planning of dosage forms and regimens.

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The Assay for Tetracycline HCl and Chlortetracycline HCl in Tetracycline HCl

By MASON H. WOOLFORD, JR., and FORTUNATO S. CHICCARELLI

A procedure is described for the determination of tetracycline[1] or for small amounts of chlortetracycline[2] in tetracycline. The procedure is based upon the changes in the spectrophotometric absorption in acid and basic aqueous media. Chlortetracycline is radically changed in basic medium while tetracycline is relatively stable. This is the basis of the assay for tetracycline. The proportional difference of the extinction coefficients of the sample in acidic and basic solutions to those established for standard materials is due to chlortetracycline. This is the basis of the assay for chlortetracycline.

To DETECT the presence of small concentrations of chlortetracycline in tetracycline, an analytical procedure was required. When this concentration is very small, fluorescence of the chlortetracycline in a basic aqueous medium (the method by which this compound is customarily determined) is completely quenched by the comparatively large proportion of nonfluorescing tetracycline. However, it was noted that under the conditions for the fluorometric analysis of chlortetracycline, solutions of both antibiotics became intensely yellow. Further investigation showed that the yellow color of chlortetracycline faded rapidly while that of tetracycline was relatively stable. It was decided to investigate this difference in behavior.

EXPERIMENTAL

A solution of tetracycline containing 10.64 mcg./ml. was prepared in 0.25 *N* NaOH. A yellow color was developed which had an absorption maximum at 380 mμ. The effect of time on the stability of this color is shown in Table I.

TABLE I

Time, Minutes	Absorbance at 380 mμ
2	0.398
4	0.396
6	0.395
8	0.393
10	0.390
12	0.388

As there is a definite decrease in the color produced, the absorbance six minutes after the addition of sodium hydroxide was arbitrarily chosen for use in the analytical procedure subsequently developed. A concentration curve was established at 380 mμ. Under these conditions the absorptivity $E(1\%,1\text{ cm.})$ of tetracycline HCl is 372.0.

In 0.25 *N* NaOH, the color developed by chlortetracycline disappears quite rapidly. Table II shows that the interference of chlortetracycline with tetracycline at 380 mμ is negligible.

From the above it can be seen that tetracycline HCl can be determined accurately and without interference in the presence of more than twenty per cent chlortetracycline HCl.

In 0.1 *N* HCl, tetracycline has a maximum absorbance at 355 mμ. Chlortetracycline, in the same medium, has an absorption maximum at

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[1] The trademark of Lederle Laboratories Division, American Cyanamid Company, for tetracycline is Achromycin®.

[2] The trademark of Lederle Laboratories Division, American Cyanamid Company, for chlortetracycline is Aureomycin®.

TABLE II

Concn. per 100 ml. 0.25 *N* NaOH			Absorbance at 380 mμ
1500 mcg. TC[a]	+	15 mcg. CTC[b]	0.557
1500 mcg. TC	+	30 mcg. CTC	0.556
1500 mcg. TC	+	45 mcg. CTC	0.557
1500 mcg. TC	+	60 mcg. CTC	0.557
1500 mcg. TC	+	150 mcg. CTC	0.557
1500 mcg. TC	+	300 mcg. CTC	0.557
1500 mcg. TC	+	400 mcg. CTC	0.557

[a] TC—tetracycline HCl. [b] CTC—chlortetracycline HCl.

367.5 mμ and would therefore interfere in the spectrophotometric determination of tetracycline. However, since chlortetracycline is rapidly destroyed in the basic medium and tetracycline is relatively stable, a differential spectrophotometric method is possible in acid and basic media for the determination of chlortetracycline in tetracycline. It had been observed (see Table III) that there is an increase in absorbance with time for chlortetracycline in an acid medium so that readings are again taken at six minutes.

TABLE III

Time, Minutes	Absorbance at 355 mμ
2.0	0.287
4.0	0.292
6.0	0.297
8.0	0.298
10.0	0.298
12.0	0.298

The concentration curves of chlortetracycline HCl and tetracycline HCl at 355 mμ were then determined. All absorbances were taken six minutes after the addition of acid. The final concentration of acid was 0.1 *N* HCl. Under these conditions the absorptivity of chlortetracycline HCl was found to be 182.6 and the absorptivity of tetracycline HCl was found to be 303.2.

Using the above data the following method has been developed.

Assay Procedure.—Transfer an accurately weighed sample of about 100 mg. of tetracycline HCl to a liter volumetric flask. Add about 100 ml. of water and shake to dissolve. After solution is complete make to volume with water.

Transfer exactly 15 ml. to a 100-ml. volumetric flask. Add 70 ml. of water and exactly 5 ml. of 5 *N* NaOH and make to volume with water. Exactly six minutes after the addition of sodium hydroxide, determine the absorbance on a suitable spectrophotometer at 380 mμ using water as a blank.

Transfer another accurately measured 15-ml. portion to a 100-ml. volumetric flask. Add 70 ml. of water and exactly 10 ml. of 1 *N* HCl and make to volume with water. Exactly six minutes after the addition of acid, determine the absorbance at 355 mμ using water as a blank.

Calculation: Per cent tetracycline HCl:

$$\frac{(\text{Absorbance at } 380\ m\mu)}{372.0^{3}} \times 100 \times \frac{1000}{15} \times \frac{1}{\text{Wt. of sample}} = \%\ \text{tetracycline HCl}$$

Per cent chlortetracycline HCl:

$$\frac{(\text{Absorbance at } 355\ m\mu) - \dfrac{(\text{Absorbance at } 380\ m\mu \times 303.2^{4}/372.0^{3})}{182.6^{5}}}{} \times 100 \times \frac{1000}{15} \times \frac{1}{\text{Wt. of sample}} = \%\ \text{chlortetracycline HCl}$$

SUMMARY

1. Tetracycline under basic conditions has an absorption maximum at 380 mμ. This is used as the basis for the control assay of tetracycline HCl even in the presence of twenty per cent chlortetracycline.[6]

2 Tetracycline under acid conditions has an absorption maximum at 355 mμ. However, this cannot be used as a measure of the tetracycline content because chlortetracycline if present under these conditions would be additive.

3. By converting the 380 mμ absorbance to the equivalent 355 mμ absorbance, the difference between this and the actual 380 mμ reading is due to chlortetracycline.[7]

[3] Absorptivity of tetracycline HCl at 380 mμ in 0.25 *N* NaOH.
[4] Absorptivity of tetracycline HCl at 355 mμ in 0.1 *N* HCl.
[5] Absorptivity of chlortetracycline HCl at 355 mμ in 0.1 *N* HCl.
[6] This method is now incorporated as the control assay for tetracycline HCl in the United States Pharmacopeia XV.
[7] This method for the determination of chlortetracycline HCl in tetracycline HCl is now incorporated in "Assay Methods of Antibiotics; A Laboratory Manual," by Dr. Donald C. Grove and Dr. William A. Randall, published by Medical Encyclopedia, Inc., 1955.

Evaluation of an Iodonium Compound as an Antiseptic Agent*

By WARREN E. ENGELHARD and ALBERT G. WORTON

A wide variety of iodonium compounds have been prepared and synthesized within the past 25 years. Investigations however have been centered primarily around the synthesis and preparation of new iodonium products and only limited data are available concerning the evaluation of their biological activity. This is rather surprising since the iodone is an integral part of these compounds, and since they are strong bases which form stable salts when neutralized with acids, and as such behave as do the quaternary ammonium compounds. The skin antiseptic, fungistatic, and bacteriostatic effectiveness of bis(dichlorophenyl) iodonium chloride was investigated. Results indicate this compound can be used as a dermatological pharmaceutical, since it markedly reduced the transient and resident microflora of the skin. Furthermore, a 1.5% solution showed fungistatic activity in dilutions as high as 1:1000. Similar activity was observed with the gram-positive bacteria, however, at higher dilutions while a more moderate yet effective inhibition was observed with the gram-negative bacteria.

NUMEROUS CHEMICALS have been evaluated for their skin degerming effectiveness (1). One, bis (2-hydroxy-3,5,6-trichlorophenyl)methane,[1] (2), exhibits marked antibacterial properties, while the halogenated phenols (3, 4) leave much to be desired. The iodonium compounds, however, encompass substances investigated more recently. Some show promise as antiseptic agents (5–7).

Such observations also show that these compounds are relatively stable and can be kept in acidic and neutral solutions in amber-colored bottles for long periods of time. In alkaline solutions, however, phenols and halogenated derivatives are formed.

Water-soluble studies indicate that iodonium sulfates and chlorides are more readily soluble than the iodides and bromides.

Although Willgerodt (8) was the first to note iodine as a polyvalent ion, Hartman and Meyer (9) were the first to detect the iodonium compounds. Unfortunately, the early work concerned primarily synthesis of different iodonium compounds from the standpoint of valence studies rather than evaluation of biological activity. Limited data are available however for some of these compounds; for example, the bacteriostatic range of diphenyl iodonium chloride (5). This compound inhibited virulent *Mycobacterium* at high dilutions, while lower dilutions were necessary for the gram-positive cocci and gram-negative rods. An intermediate range of bacteriostasis was observed for anaerobes and molds. Reynolds (6) obtained similar bacteriostatic limits, and also observed that the lower concentrations were bactericidal for the above organisms.

Bis-*p*-chlorophenyliodonium sulfate, bis-*p*-bromophenyliodonium iodide, bis-*p*-iodophenyliodonium iodide, bis-*p*-chlorophenyliodonium iodide and diphenyliodonium iodide have also recently been investigated (7). These powdered compounds displayed bacteriostatic activity against the micrococci (10). With saturated aqueous solutions however only bis-*p*-chlorophenyliodonium sulfate displayed bactericidal efficiency. Similar activity was observed in an acetone-alcohol solvent within one minute against the gram-negative bacteria and the micrococci, while for spore formers a bactericidal and sporocidal effect occurred within four hours at 37°.

Of the other iodonium salts investigated and found to be effective against gram-negative organisms (11), the highest bactericidal efficiency was displayed by bis (3,4-dichlorophenyl) iodonium chloride. Finally, iodonium chlorides have also been evaluated by the spore germination screening test and given A and AA ratings against the pathogens *Alternaria oleracea* and *Sclerotinia fructicola*. The ratings scale of A and AA being LD_{50} = 1-10 p. p. m. and LD_{50} = < 1 p. p. m. respectively (13).

It is evident therefore that these iodonium compounds display biological activity. For this reason a more detailed fungicidal, skin antiseptic, and bactericidal study was undertaken with one of the more effective of these compounds, bis (chlorophenyl) iodonium chloride-Iodonium 235.[2]

MATERIALS AND METHODS

The cutaneous antiseptic activity of Iodonium 235 was determined by the Cade modification (12)

* Received November 14, 1955, from the Department of Bacteriology, University of Nebraska, Lincoln.

[1] G-11 (Brand of hexachlorophene) Trademark, Sindar Corp.

[2] Trademark: Warren-Teed Pharmaceutical Corp.

of the Price (1) basic procedure. In a typical series, 6 participants were used to test the iodonium compound. The group was chosen on the basis of willingness to cooperate, reliability, and a large predetermined bacterial count. Each participant used 5 enamel pans containing one liter of sterile tap water. A surgical brush and a bland brand of hand soap were used. The scrubbing procedure involved: soap scrubbed into brush, 15 seconds; both hands scrubbed up to wrists, 45 seconds; (sterile forearm sleeves were used), both hands rinsed in the sterile tap water, 15 seconds. The same procedure was repeated in the 4 remaining basins. Using a modified plating technique[3] 1 and 0.1 ml. amounts from the 1st, 3rd and 5th basins were inoculated into 6 plates of tryptose glucose extract agar (TGE), and incubated at 37° for forty-eight hours. The number of colonies were then recorded.

Following the control initial wash, the members washed their hands twice daily, for two minutes, morning and evening, with 2.5 ml. of the Iodonium preparation. This procedure was followed for 3 days, before repeating the scrubbing procedure with the bland soap. This was repeated on the morning of the 4th, 8th, 12th, and 16th day, before the participants washed their hands for that day. Colonies were counted and the reduction of skin microflora recorded as the log of numbers on the initial, and on the 4th, 8th, 12th, and 16th day after using Iodonium 235. A similar procedure using a standard antiseptic solution of hexachlorophene and a bland soap are included as controls.

In addition six bacteria and 5 pathogenic fungi were tested to determine the bacteriostatic and fungistatic properties of Iodonium 235. Fungistatic activity was determined by placing penicillin assay disks in different dilutions of Iodonium 235 for a ten-minute period. The disks were then dried on filter paper for one hour and then placed on blood and Sabouraud's agar plates containing 10% human serum, which was surface seeded thirty-six hours earlier with the 5 pathogenic fungi. Zones of inhibition were then recorded.

Bacteriostatic determinations were observed by preparing dilutions of hexachlorophene and Iodonium 235 in 10 ml. of brain heart infusion broth containing 10% human serum. Each bacterial suspension was adjusted spectrophotometrically and 0.2 ml. inoculated into each of the dilutions. The tubes were incubated for forty-eight hours at 37° after which time they were subcultured into the same media, and after two days observed for turbidity.

DISCUSSION AND RESULTS

The skin entertains both transient and resident microflora. The former are accumulated from our daily environmental contacts, and can be readily removed with a bland soap. The latter however, are more deeply implanted. They parasitize the deeper pores, crevices, hair follicles, and unlike the transients are not readily removed. Furthermore, since they are imbedded they resist the action of mild antiseptics. Therefore, if we perform uniform consecutive washings of the skin with a bland soap transient bacteria are removed in a progressively decreasing order. When the washings are collected in a series of basins and log of numbers plotted, we can determine the rate of removal from the skin. An effective antiseptic soap however should, over a period of days, reduce the resident flora to the extent that a much smaller number of microorganisms are present in the rinse waters. Although the Price method does not give an absolute measure of the number of microorganisms present on the skin, we can assume that a small number of bacteria removed by the scrub technique is indicative of a small bacterial skin population.

Results obtained with diphenyliodonium chloride indicate a bacterial reduction of 90 to 95 per cent of the initial count when this compound was used daily over a period of 16 days.

Therefore (Fig. 1), after removal of the transient microorganisms, the daily application of Iodonium

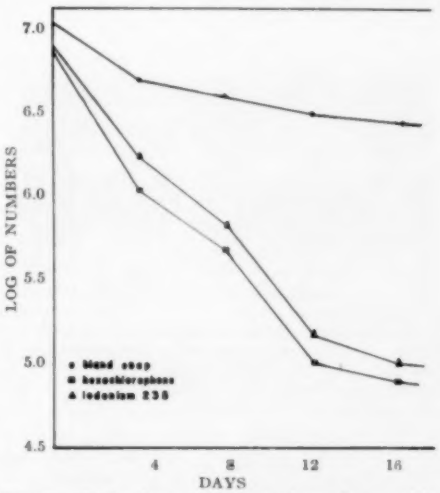

Fig 1.—Reduction of resident bacteria on skin.

235 effectively reduced the imbedded resident flora. The numbers removed are comparable to that achieved by using the standard surgical scrub technique. Our objective therefore to reduce the resident flora and prevent the reestablishment of the many transient microflora that parasitize the skin has been achieved. Furthermore, the residual effect of this iodonium compound provides continued protection. Preliminary experiments in our laboratories indicate that once Iodonium 235 has been used repeatedly the time required for the microflora to become re-established may be as long as 4 to 7 days. In addition, application of the standard patch test indicates this substance to be nonirritating and nonsensitizing when continuously applied over a seventy-two hour period.

Removal therefore, of this resident flora, which includes numerous gram-positive organisms, prevents the possibility of frequent skin infections which plague adolescents in particular. Resident microflora also metabolize certain constituents of per-

[3] The basin bacteria were introduced onto a thin layer of solidified TGE agar and 15 ml. of liquid TGE agar was added and the plate rotated to disperse the cells.

spiration and fatty substances which contribute to offensive body odors. Furthermore, the necessity of removing microflora, particularly in the fields of nursing, surgery, and food handlers is quite apparent.

Finally, as a fungistatic agent a 1.5% solution inhibited the growth of *Epidermophyton floccosum*, *Microsporum canis*, and *Trychophyton mentagrophytes* at dilutions of 1:1000, *Microsporum audouini* at 1:100, and *Audida albicans* at 1:10 (Fig. 2 and Table I).

As a basteriostatic agent a 1.5% solution prevented the growth of the gram-positive bacteria *Micrococcus pyogenes var. aureus* at dilutions of 1:100,000 and the spore forming *Bacillus subtilis* at dilution of 1:10,000. The gram-negative organisms *Escherichia coli*, *Proteus vulgaris*, and *Salmonella typhosa* were inhibited at dilutions ranging from 1:5000 to 1:15,000. The iodonium salt was least effective against *Pseudomonas aeruginosa* which required dilutions lower than 1:1000 to prevent growth (Table II).

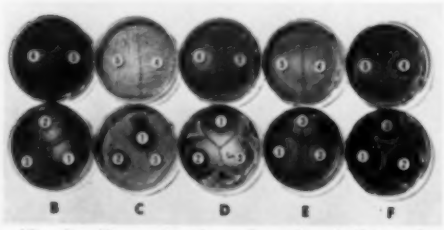

Fig. 2.—Demonstration of antifungicidal action of Iodonium 235 (1.5%) by disk method on growth of (B) *Epidermophyton floccosum*, (C) *Audida albicans*, (D) *Microsporum canis*, (E) *Microsporum audouini*, and (F) *Trychophyton mentagrophytes* at dilutions of (1) undiluted 1.5%, (2) 1:10, (3) 1:100, (4) 1:1000, and (5) 1:10,000.

TABLE I.—FUNGISTATIC ACTIVITY OF 1.5% BIS (CHLOROPHENYL) IODONIUM CHLORIDE

Microorganism	Zones of Inhibition in Mm. Dilutions				
	Concn.	10	100	1000	10,000
Epidermophyton floccosum	15	14	8	4	...
Audida albicans	5	3	..	..	...
Microsporum canis	15	15	10	5	...
Microsporum audouini	12	9	7	..	...
Trychophyton mentagrophytes	15	13	9	5	...

TABLE II.—HIGHEST BACTERIOSTATIC DILUTION IN VITRO-(x1000)

Microorganism	Bis (chlorophenyl) iodonium chloride 1.5% in detergent base	3% hexachlorophene in soap solution
Escherichia coli	5	2
Proteus vulgaris	15	10
Salmonella typhosa	10	10
Pseudomonas aeruginosa	1	1
Micrococcus pyogenes var. aureus	100	100
Bacillus subtilis	10	10

SUMMARY

The skin antiseptic, fungistatic, and bacteriostatic effectiveness of bis (dichlorophenyl) iodonium chloride was investigated. Results indicate this compound can be used as a dermatological pharmaceutical, since it markedly reduced the transient and resident microflora of the skin. Furthermore, a 1.5% solution showed fungistatic activity in dilutions as high as 1:1000. Similar activity was observed with the gram-positive bacteria, however, at higher dilutions, while a more moderate yet effective inhibition was observed with the gram-negative bacteria.

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Polarographic Determination of Pyrazinamide Serum and Plasma Concentrations in the Presence of Isoniazid*

By GLENN R. SVOBODA†

A simple polarographic method for the quantitative determination of pyrazinamide (pyrazine monocarboxyl amide) in human blood serum or plasma is described. The method utilizes a protein-free filtrate obtained by the use of uranyl acetate. Isoniazid (pyridine-3-carboxylic acid hydrazide) does not interfere. Pyrazinoic acid (pyrazine monocarboxylic acid) and pyrazinamide give a single wave, but the individual concentrations may be determined by means of a minimum error potential analysis. The sample can be used directly for isoniazid analysis after pyrazinamide determination.

A RENEWED interest in the use of pyrazinamide in the chemotherapy of tuberculosis has followed reports that the combined therapy of tuberculosis with isoniazid and pyrazinamide is equal or superior to other treatment regimens (1, 2, 3). In following the clinical progress of patients on specific chemotherapy it is desirable to determine, at appropriate intervals, the serum concentration of the chemotherapeutic agents being used. Since serum biological assay procedures are not easily applied in instances where two or more chemotherapeutic agents are being used simultaneously, there is a need for an accurate and rapid chemical method.

A reported colorimetric method requires the use of ion exchange columns (4). The polarographic method described below eliminates such requirements, thereby lending itself more readily to routine clinical analysis. By means of the minimum error potential analysis (5) this polarographic method is also able to distinguish between pyrazinamide and pyrazinoic acid, one of the possible metabolic products, since the amide is readily hydrolyzed in dilute alkali (4).

In the polarographic analysis of a mixture of two reversibly reducible compounds, whose half-wave potentials lie in close proximity to each other, the polarograms do not show sufficiently distinct changes in slope to enable the evaluation of the diffusion current corresponding to each of the individual components. The application of a mathematical interpretation of such a polarogram enables the calculation of the proportionate amounts of each of the components. To accomplish this it is necessary only to determine the potential of minimum error, and measure the current at the potential of minimum error and the current equivalent to the total diffusion current.

EXPERIMENTAL

Materials.—Pyrazine monocarboxyl amide and pyrazine monocarboxylic acid[1] were of a quality designated for investigational use. All other materials used were of reagent quality.

Polarography of Pyrazinamide and Pyrazinoic Acid in Aqueous Solution

To determine the polarographic behavior of pyrazinamide and pyrazinoic acid, solutions of 100 mcg./cc. were prepared and mixed with equal quantities of a universal buffer which contained 0.1 *M* phosphoric, acetic, and boric acids and 0.5 *M* potassium chloride. Various amounts of 1 *M* sodium hydroxide were added to aliquots of the buffer solution to obtain the desired *p*H values. All *p*H measurements were made with a Beckman Model H-2 *p*H meter calibrated at *p*H 4.0 and 7.0 with standard buffers. Polarograms were recorded with a Leeds and Northrup Type E Electrochemograph in conjunction with an H-type cell and a conventional dropping mercury electrode. No attempt was made to obtain precise temperature control since it was noted that room temperature varied only from 24.5° to 25.5°. Oxygen was removed from the test solutions by bubbling for five minutes with nitrogen purified by passage through Fieser's (6) solution. Capillary characteristics at −0.3 v. *vs.* saturated calomel electrode (S.C.E.) showed $m^{2/3}t^{1/6}$ equal to 2.4721.

Appropriate dilutions of a stock solution of pyrazinamide were prepared in the concentration range of 20 to 300 mcg./3 cc. at 20-mcg. increments. Pyrazinoic acid dilutions were prepared in the concentration range of 10 to 100 mcg./cc. at 10-mcg. increments. The cell solution in all cases consisted of 3 cc. of dilution mixed with 3 cc. of aqueous universal buffer. Pyrazinamide solutions were run between −0.30 and −0.60 v. *vs.* S.C.E. Solutions of the acid were run between −0.20 and −0.50 v. *vs.* S.C.E.

* Received November 3, 1955, from the Research and Development Service, Fitzsimons Army Hospital, Denver, Col.

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[1] Kindly supplied by Dr. Charles E. Lyght of Merck & Company, Inc.

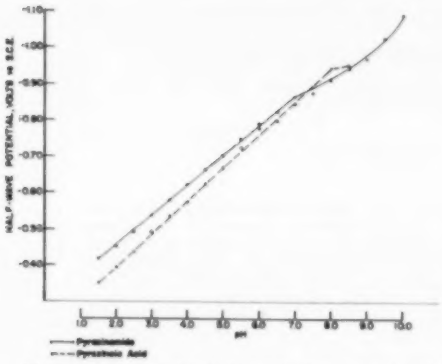

Fig. 1.—Relationship between half-wave potential and *p*H for pyrazinamide and pyrazinoic acid in a universal buffer.

Pyrazinamide was found to exhibit a single, well-defined polarographic wave in the *p*H range of 1.5 to 7.0, after which it no longer exhibited a linear relationship between half-wave potential and *p*H (Fig. 1). The curve for pyrazinoic acid closely parallels that of the amide. The wave of the acid also becomes poorly defined in basic solution.

A *p*H of 1.5 was chosen because a good resolution of the pyrazinamide-pyrazinoic acid wave and the two succeeding waves of isoniazid (7) was obtained (Fig. 2). At this *p*H, pyrazinamide alone exhibited a half-wave potential of −0.417 v. *vs.* S.C.E., whereas that of the acid alone was −0.362 v. *vs.* S.C.E. in aqueous solution.

The linear relationship between diffusion current and concentration for both pyrazinamide and pyrazinoic acid is shown in Fig. 3. Linearity is a prerequisite for the application of the minimum error potential analysis. The height of the mercury column above the tip of the capillary must remain constant for the relationship to hold.

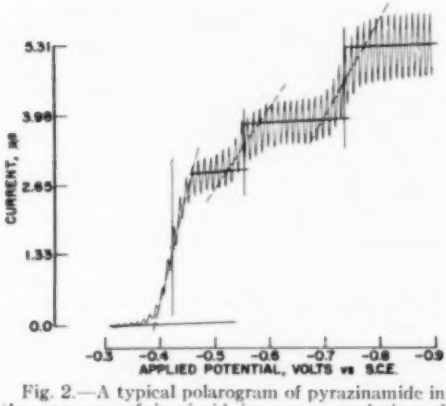

Fig. 2.—A typical polarogram of pyrazinamide in the presence of isoniazid in aqueous solution of *p*H = 1.5 (37.5 mcg. pyrazinamide/cc.; 14.3 mcg. isoniazid/cc.).

Minimum Error Potential Analysis of Aqueous Solutions.—Providing the condition of noninteraction of the two components of a binary mixture is met, the fundamental equation of Heyrovský and Ilkovič is valid for such a mixture. This equation can then be presented in the form of Eq. 1:

$$(A) = \frac{(id_{AB}K_B - i_{AB})}{(K_B - K_A)(K'_A)} \text{ and } (B) = \frac{(i_{AB} - id_{AB}K_A)}{(K_B - K_A)(K'_B)} \quad (1)$$

Where A and B are the concentrations of the two components, id_{AB} is the total diffusion current assuming additivity, i_{AB} is the current reading at the predetermined potential of minimum error, K'_B and K'_A are the constants derived for each of the pure compounds if the linear relationship between diffusion current and concentration holds for each

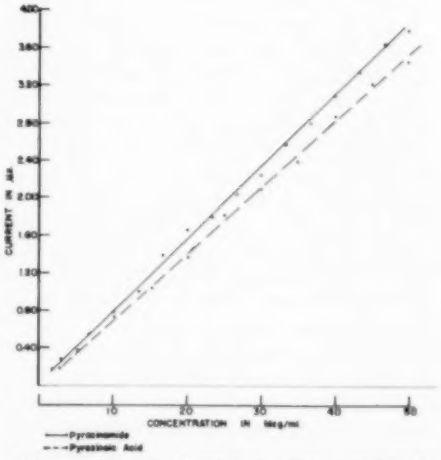

Fig. 3.—Diagram showing the linear relationship between current and concentration of pyrazinamide and pyrazinoic acid in aqueous solution at *p*H of 1.5. (All points are the average values of duplicate determinations.)

component, and K_B and K_A are the constants at any one potential, in this case, the potential of minimum error. A complete derivation may be found in the work of Frisque, *et al.* (5).

When the same electron change is undergone by both components ($n_A = n_B$), it follows that the minimum error potential is defined by Eq. 2:

$$E = {}^1/_2(E_{1/2B} + E_{1/2A}) \quad (2)$$

It was found that the reduction of both the acid and the amide was a two electron reaction, giving a minimum error potential in aqueous solution of −0.390 v. *vs.* S.C.E.

Additivity of the individual diffusion currents of the acid and amide when mixed was found within the limits of ±0.08 μa over the entire concentration range studied.

Tables I and II give the experimental values employed on synthetic mixtures. Table III gives the

single potential constants for each component present in mixtures and the difference between the constants as a function of applied potential.

TABLE I.—EXPERIMENTAL VALUES FOR PYRAZINOIC ACID EMPLOYED IN CALCULATIONS ON SYNTHETIC MIXTURES

Concentration, mcg./ml.	$E_{1/2}$	i_d Pyr. Acid (μa)	K' Pyr. Acid
50	−0.364	3.46	0.0692
40	−0.363	2.87	0.0714
30	−0.362	2.10	0.0700
20	−0.361	1.37	0.0685
10	−0.360	0.73	0.0730
	Av. −0.362		0.0704 = K'_B

TABLE II.—EXPERIMENTAL VALUES FOR PYRAZINAMIDE EMPLOYED IN CALCULATIONS ON SYNTHETIC MIXTURES

Concentration, mcg./ml.	$E_{1/2}$	i_d Pyr. (μa)	K' Pyr.
50	−0.417	3.78	0.0756
40	−0.418	3.09	0.0773
30	−0.415	2.24	0.0747
20	−0.416	1.66	0.0830
10	−0.417	0.78	0.0780
	Av. −0.417		0.0777 = K'_A

TABLE III.—CONSTANT[a] FOR EACH COMPONENT PRESENT IN MIXTURES AND DIFFERENCE BETWEEN CONSTANTS AS A FUNCTION OF APPLIED POTENTIAL

Applied Potential, v.	K_B	K_A	$(K_B - K_A)$
−0.330	0.077	0.001	0.076
−0.360	0.465	0.014	0.451
−0.380	0.806	0.052	0.754
−0.390	0.901	0.107	0.794
−0.400	0.952	0.208	0.744
−0.430	0.990	0.901	0.089
−0.460	1.000	0.971	0.029

[a] Single potential constant

$$K = 1/[1 + 10\,(E - E_{1/2})\,n/0.059]$$

K_A = K (Pyrazinamide)
K_B = K (Pyrazinoic acid)
n = 2 for both compounds
$E_{1/2}$(Pr) = −0.362
$E_{1/2}$(Pyracid) = −0.417

The necessity of determining the potential of minimum error is obvious. Any small error in the determination of either K_A or K_B will cause a relatively smaller error in the concentrations (A) and (B) if the difference between the two constants is large. Thus, the current i_{AB} should be measured at the potential at which the quantity $(K_B - K_A)$ is at a maximum.

Since $(K_B - K_A)$ is a maximum when i_{AB} is evaluated at an applied potential of −0.039 v. *vs.* S.C.E., the differences between the theoretical and experimental values for the amount of pyrazinamide and pyrazinoic acid present in each mixture should be at a minimum value when i_{AB} is measured at this potential. Synthetic mixtures of pyrazinamide and pyrazinoic acid were prepared at a total concentration of 100 mcg./ml. with ratios of 10:90 to 90:10 at 10-mcg. increments for each component. Three cubic centimeters of each solution was mixed with 3 cc. of universal buffer at *p*H 1.5. (Final concentration ratios at 5:45 to 45:5 at 5-mcg. increments.) All solutions were treated as above and polarographed between −0.20 and −0.55 v. *vs.* S.C.E. Table IV gives a comparison of the theoretical and experimental results obtained on such synthetic mixtures.

Such a method for the determination of binary mixtures employing the described procedure has obvious limitations. The resulting error in experimentally determined values for the amount of each component present becomes larger as the ratio of the half-wave potentials of the two components approaches unity. It must be further emphasized that since the minimum error potential method assumes a single clearly defined half-wave potential for each component in the applied potential range of interest, the analysis, therefore, will not hold when a stepwise exchange of electrons occurs for one of the components at nearly similar voltages.

Selection of a Protein Precipitant.—Polarographic determinations made directly on serum or plasma showed the wave of pyrazinamide to be absent. Associated with this behavior was the observation that the individual mercury droplets did not coalesce in the bottom of the electrolysis cell. Recurrent observation of these phenomena was attributed to incomplete protein removal.

Since the preparation of an extract was considered to be too time-consuming for routine analysis, a number of protein-precipitating reagents were investigated to find one which would not give an interfering wave in the potential range of −0.2 v. to

TABLE IV.—THEORETICAL AND EXPERIMENTAL VALUES FOR PYRAZINAMIDE AND PYRAZINOIC ACID IN SYNTHETIC MIXTURES OF THE TWO COMPOUNDS

Pyrazinamide			Pyrazinoic Acid		
Theor., mcg./ml.	Exptl., mcg./ml.	Error, %	Theor., mcg./ml.	Exptl., mcg./ml.	Error, %
0.0	3.9	...	50.0	49.0	− 2.0
5.0	5.9	+18.0	45.0	42.3	− 6.0
10.0	11.0	+10.0	40.0	40.6	+ 1.5
15.0	11.3	−24.7	35.0	38.6	+10.3
20.0	21.2	+ 6.0	30.0	30.0	0.0
25.0	25.2	+ 0.8	25.0	25.9	+ 3.6
30.0	32.2	+ 7.3	20.0	20.2	+ 1.0
35.0	36.7	+ 4.9	15.0	9.9	−34.0
40.0	40.0	0.0	10.0	9.3	− 7.0
45.0	40.8	− 9.3	5.0	6.8	+36.0
50.0	48.0	− 4.0	0.0	0.7	...
	Av. rel. error	8.5		Av. rel. error	10.1

TABLE V.—POLAROGRAPHIC CHARACTERISTICS OF PROTEIN PRECIPITANTS INVESTIGATED

Compound	Characteristics
Trichloroacetic acid (20%)	An interfering catalytic-type wave between −0.3 v. and −0.8 v. *vs.* S.C.E.
Tungstic acid (10%) (sodium salt with sulfuric acid)	Wave of $E_{1/2}$ at −0.69 v. *vs.* S.C.E., with increasing residual current noted starting at −0.3 v. *vs.* S.C.E., which covered the pyrazinamide wave at low concentrations.
Absolute ethanol	Incomplete protein precipitation. (Hg droplets do not coalesce.)
Metaphosphoric acid (20%)	Incomplete protein precipitation. (Hg droplets do not coalesce.)
Tannic acid (20%)	Excellent precipitant; mercury droplets coalesce; pyrazinamide wave has $E_{1/2}$ shifted to −0.52 v. *vs.* S.C.E.; low residual current and good wave obtained.
Uranyl ion (saturated solution as the acetate)	Excellent precipitant; Hg droplets coalesce; low residual current and good wave obtained.

−0.6 v. *vs.* S.C.E. The commonly used protein precipitants were found to interfere in this range. Table V lists the protein precipitants investigated and the polarographic characteristics observed.

The results presented in Table V indicate tannic acid to be as efficient a protein precipitant as uranyl acetate. However, the excess tannic acid prevented the subsequent use of vanillin for isoniazid determination (8, 9). Uranyl acetate was found to be a suitable substitute for trichloroacetic acid, necessitating only the preparation of new standard curves. The use of this procedure had the added advantage of using a minimum of serum for determination of more than one chemotherapeutic agent.

Polarography of Pyrazinamide in Protein-Free Plasma or Serum Centrifugates

A stock solution of 100 mg. pyrazinamide/100 cc. plasma or serum was prepared. Dilutions of 10 to 150 mcg./cc. at 10-mcg. increments were prepared by further dilution with pooled plasma or serum. Standard curves were prepared for an original serum or plasma volume of 1 cc. and 3 cc. The samples were then treated in the following manner.

(*a*) One cubic centimeter of serum or plasma (3 cc.) was mixed with 1 cc. (3 cc.) of saturated uranyl acetate in a 15-cc. centrifuge tube and centrifuged for twenty minutes at 1800 G (10).

TABLE VI.—COMPARISON OF THEORETICAL RECOVERIES OF SERUM AND PLASMA PYRAZINAMIDE TO ACTUAL RECOVERIES[a]

Orig. Concn., mcg./cc.	1-cc. Sample			3-cc. Sample		
	Theor., mcg./cc.	Found, mcg./cc.	Recovery, %	Theor., mcg./cc.	Found, mcg./cc.	Recovery, %
10	1.7	0.3	17.7	4.8	2.0	41.7
20	3.5	1.0	28.6	9.7	4.3	44.3
30	5.2	1.7	32.6	14.5	6.7	46.2
40	6.9	2.3	33.4	19.4	9.7	50.0
50	8.7	3.3	37.9	24.2	11.3	46.7
60	10.4	4.0	38.4	29.0	14.7	50.7
70	12.1	5.0	41.7	33.9	16.7	49.3
80	13.8	6.0	43.5	38.8	18.3	47.2
90	15.6	7.0	44.9	43.6	21.0	48.1
100	17.3	8.0	46.2	48.4	22.7	47.0
110	19.0	8.7	45.8	53.2	26.7	50.2
120	20.8	9.3	44.7	58.1	29.3	50.4
130	22.5	9.7	43.1	62.9	31.7	50.4
140	24.2	10.7	44.2	67.8	34.0	50.2
150	26.0	11.3	43.5	72.6	35.3	48.6
		Av. recovery =	38.4			48.0

[a] Theoretical calculated as original concentration/final volume for 1-cc. and 3-cc. samples. Average final volumes are 5.8 cc. and 6.2 cc. respectively.

TABLE VII.—COMPARISON OF THEORETICAL AND ACTUAL RECOVERIES OF PYRAZINAMIDE FROM A 3-CC. SAMPLE[a]

Orig. Concn., mcg./cc.	Theor. (Orig./6.2 cc.), mcg./cc.	Found, mcg./cc.	Recovered, %	Theor. 1st Wash (Theor.—Found/5.9 cc.), mcg./cc.	Found, mcg./cc.	Recovered, %	Total Recovered, %
10	4.8	2.1	43.8	2.9	1.0	34.4	63.0
30	14.5	6.4	44.1	8.5	3.7	43.5	68.3
50	24.2	10.6	43.8	14.3	4.3	30.1	60.7
100	48.4	21.5	44.4	28.3	8.9	31.4	61.9
150	72.6	34.9	48.1	39.6	13.9	35.1	66.3

[a] Theoretical calculated as in Table VI; theoretical of 1st wash calculated as difference between theoretical and found for original protein precipitation.

TABLE VIII.—ANALYSIS OF PATIENT SERUM POLAROGRAMS[1]

Patient No.	Concn. in mcg./ml. at Designated Hour After Oral Administration																	
	1 hr.			2 hr.			3 hr.			7 hr.			8 hr.					
	PZA (MEP)	PZA (Tot. Wave Ht.)	Pyr. Acid (MEP)	PZA (MEP)	PZA (Tot. Wave Ht.)	Pyr. Acid (MEP)	PZA (MEP)	PZA (Tot. Wave Ht.)	Pyr. Acid (MEP)	PZA (MEP)	PZA (Tot. Wave Ht.)	Pyr. Acid (MEP)	PZA (MEP) (a)	PZA (MEP) (b)	PZA (Tot. Wave Ht.) (a)	PZA (Tot. Wave Ht.) (b)	Pyr. Acid (MEP) (a)	Pyr. Acid (MEP) (b)
1	13.4	13	0.0	...[b]	..	...	...	..	...	12.9	13	0.6	15.9	..	15	..	0.0	...
2	53.9	54	1.6	47.4	48	1.6	33.2	40	5.6	28.9	32	2.8	35.3	31.0	35	34	0.6	3.4
3	25.4	25	0.6	33.2	34	1.2	19.8	21	1.6	5.6	6	0.0	17.7	0.0	17	0	0.6	0.0
4	24.6	24	0.0	...	..	...	...	..	...	...	..	...	4.3	...	4	..	0.0	...
5	44.4	46	1.6	98.8	92	1.2	60.4	70	4.0	58.6	54	0.0	51.7	78.4	51	77	0.0	0.0
6	32.8	32	0.0	...	..	...	24.6	30	4.4	19.0	21	1.2	19.0	20.7	19	22	0.6	1.2
7	33.2	34	1.6	34.5	37	2.2	23.3	29	4.4	19.0	21	1.2	24.1	26.3	25	28	1.6	1.6
8	42.7	42	0.6	...	..	...	...	..	...	24.6	25	1.2	21.1	22.8	21	23	0.0	0.6
9	...	..	...	46.5	49	3.4	...	..	...	20.7	21	1.2	24.6	...	25	..	0.6	...
10	46.5	48	1.6	51.7	53	1.6	38.8	41	2.2	22.8	24	1.6	32.7	34.0	33	35	0.6	1.6
11	38.8	41	2.8	36.2	40	3.4	24.6	30	4.4	19.8	22	2.2	23.3	25.4	26	30	1.6	4.0
12	49.6	50	1.2	...	..	...	29.7	34	4.0	26.3	26	0.6	28.9	25.4	29	25	0.0	0.6
13	31.9	33	0.6	35.3	38	3.4	30.6	33	2.8	26.3	26	1.2	27.6	26.7	27	28	0.0	1.2
14	28.9	29	0.0	24.1	26	2.2	24.6	25	1.2	16.8	17	0.6	19.0	12.9	18	13	0.0	0.6
15	64.2	65	1.6	54.3	58	3.4	40.3	48	7.5	36.2	39	2.8	46.1	...	46	...	1.2	...

[a] Pyrazinoic acid calculated by the minimum error potential method. Pyrazinamide calculated both by the minimum error potential method and by assuming no hydrolysis and thus total wave height due to pyrazinamide.
[b] Missing figures due to inability to obtain serum sample.

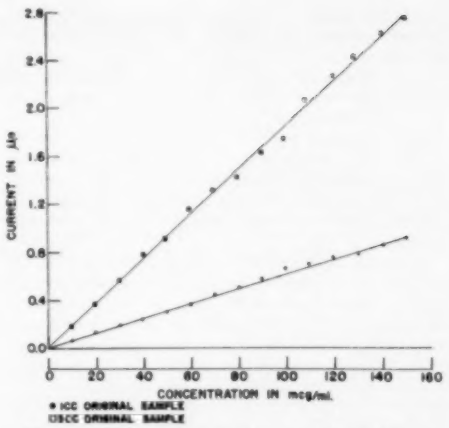

Fig. 4.—Standard curve for pyrazinamide concentration of protein-free plasma or serum centrifugation. (All points are the average values of duplicate determinations.)

(*b*) The yellow-colored centrifugate was decanted into another 15-cc. centrifuge tube and mixed with 3 cc. (both original sample volumes) of universal buffer of *p*H 1.5.

(*c*) The resulting flocculent suspension of slightly soluble alkali uranyl phosphate was centrifuged again for twenty minutes at 1800 G (10), then 2 cc. of distilled water was added to bring the total volume of clear centrifugate to more than 5 cc. Where the original sample was 3 cc., no addition of water was necessary.

(*d*) Five cubic centimeters of the clear supernatant was then pipetted into the electrolysis vessel and after deoxygenating for five minutes, the pyrazinamide concentration was determined and the sample recovered.

Although the standard curves represented in Fig. 4 were prepared from plasma, it was found that identical values were obtained with serum.

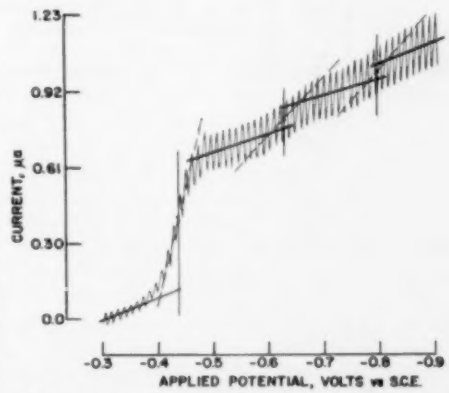

Fig. 5.—Typical polarogram of patient serum concentration of pyrazinamide, demonstrating the inability to measure isoniazid concentration polarographically (28 mcg. pyrazinamide/cc. serum; 5.2 mcg. isoniazid/cc. serum).

The advantage in using as large a plasma or serum sample volume as possible was to minimize the dilution factor. It was found necessary to obtain a filtrate volume of greater than 5 cc. for the H-type electrolysis vessel used. This volume necessitates an approximate dilution factor of 1:6 for a 1-cc. sample and 1:2 for a 3-cc. sample. Since the recoveries are of the order of 40 to 50% for a 3-cc. sample as compared to 17 to 45% for a 1-cc. sample, as shown in Table VI, this is a further indication of the desirability of using a larger serum or plasma sample.

Although the recoveries were found to be low, the consistency (±6% in the patient serum concentration range of 20 to 50 mcg./cc.) was considered adequate to give accurate results. However, further information regarding the remainder of the pyrazinamide was deemed advisable. Accordingly, another series of samples was prepared and treated as above. The original protein precipitate was then washed with 3 cc. of distilled water and recentrifuged. Three cubic centimeters of the centrifugate was then mixed with 3 cc. of universal buffer and treated as above. This was considered as the first wash. The procedure was repeated to obtain the second wash. Table VII gives the results of the additional recovery.

The second wash was found to give a poorly defined wave, which could not be accurately measured. Noncoalescence of the mercury droplets was noticed in the majority of these cases. This was attributed to protein solubility upon repeated washing. No advantage could be seen in incorporating the washing technique into the procedure, since it doubled the volume and did not result in a corresponding increase in recovery of pyrazinamide.

It might seem that a concurrent polarographic determination could also be made for isoniazid. However, comparison of the aqueous (Fig. 2) and protein-free serum-centrifugate polarograms (Fig. 4) show a marked difference in isoniazid concentration. As long as patient serum isoniazid concentrations are of the order of 0.1 to 15 mcg./ml. (9), a further dilution in obtaining a protein-free filtrate gives a final isoniazid concentration too low to measure accurately polarographically in the cited system.

Minimum Error Potential Analysis of Protein-Free Filtrates.—It must be assumed that hydrolysis of the amide as a means of detoxification *in vivo* could occur. This necessitated the determination of the potential of minimum error in a protein-free serum filtrate system. Pyrazinamide was found to exhibit a half-wave potential of −0.439 v. *vs.* S.C.E. while that of the acid was −0.383 v. *vs* S.C.E. This resulted in a minimum error potential of −0.406 v. *vs* S.C.E. A recalculation of the single potential constants K_A and K_B for a protein-free filtrate system gave values of 0.071 and 0.855, respectively. Table VIII gives the results of the analyses of patient serum polarograms.

The determination of the absolute concentration of both the amide and the acid necessitates consideration of both the dilution and recovery factors. For pyrazinamide the average recovery is 48%. The recovery of pyrazinoic acid in the concentration range of 5 to 10 mcg./cc. is 66.7%. The correction for the dilution factor for both is 6.2 cc./3 cc. Thus, the factors for the acid and the amide are 3.11 and 4.31, respectively. These are the quantities which must be multiplied by the concentration determined by the minimum error potential analysis, in order to obtain the absolute value for the concentration/cc. of patient serum.

It can be observed in Table VIII that little or none of the amide undergoes hydrolysis *in vivo* in the 1 to 8-hour period of study. For this reason, it can be assumed for clinical evaluation, that the entire wave height is due to pyrazinamide and an analysis for the acid content is not necessary. This then allows the simple comparison of experimental i_d values with a standard curve, from which pyrazinamide concentrations may be read directly.

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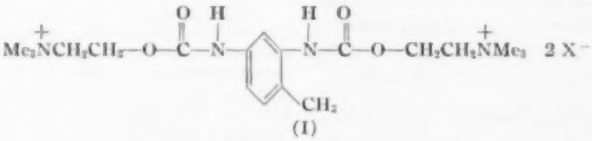
Derivatives of Arylpolycarbamic Acids of Pharmacologic Interest*

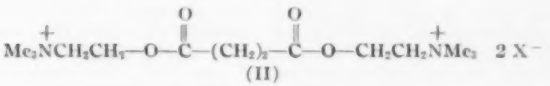
By GEORGE P. HAGER and TULLY J. SPEAKER

Urethanes and urea derivatives prepared by reaction of arylpolyisocyanates with various tertiary-amino alcohols and N,N-disubstituted-alkylenediamines, respectively, have been prepared for pharmacologic investigation. The polyquaternary salts are of particular interest in view of their structural relationship to such neuromuscular-blocking agents as succinylcholine and hexamethylene bis(carbamoylcholine).

THE AVAILABILITY of arylpolyisocyanates for the production of adhesives and foamed resins, prompted a study of their use in the preparation of arylpolycarbamates and arylpolycarbamides from amino alcohols and diamines respectively. The quaternary salts prepared from the arylpolycarbamates are of interest in tests for myoneural-blocking activity. Such compounds, e.g., *m*-tolylenebis(carbamoylcholine), I, are structurally related to the neuromuscular-blocking agents, succinylcholine, II, and hexamethylenebis(carbamoylcholine), III.



(I)



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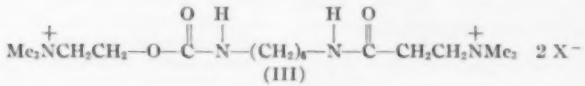


(III)

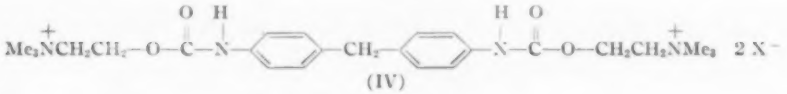
It is possible that the replacement of alkylene groups of compounds of type III with aryl groups to produce compounds of type I may engender little change in activity provided other structural determinants of activity are kept reasonably constant. This is true since a similar replacement of methylene groups in decamethylene bis(trimethylammonium) salts with aryl groups preserves the characteristic depolarizing activity of compounds of this type (1). It is anticipated that the greater duration of action described for carbamates of type III compared to that of the carboxylates of type II, may also be characteristic of the carbamates of type I. The prolonged action of the carbamates has been ascribed to their resistance to hydrolysis under the influence of enzymes (cholinesterase and pseudocholinesterase) which exert a greater effect on the rate of hydrolysis of the carboxylates (2).

It was believed that the problem of distance between cationic heads should be investigated empirically since the optimum distance in carboxylates of type II is determined by about ten intervening carbon and oxygen atoms (3) as is true also of the polymethylene bis(trimethylammonium) salts (4); while the same intramolecular dimension optimum for neuromuscular-blocking activity in carbamates of type III is determined by interposition of sixteen groups (5). The distance between cationic heads in the arylpolycarbamates was varied by use of *p,p'*-diisocyanatodiphenylmethane to produce compounds of type IV, and by use of amino alcohols homologous with choline to produce compounds of type V. Although an increase in the number of quaternary functions to three has been reported as detrimental to activity in certain myoneural-blocking agents (3), because of the availability of *p,p',p''*-triisocyanatotriphenylmethane, the effort was made to produce the corresponding tris-(carbamoylcholine) salt. However, a crystalline product could not be obtained.

* Received June 6, 1955, from the School of Pharmacy, University of Maryland, Baltimore.

Presented to the Scientific Section, A. Ph. A., Miami Beach meeting, May, 1955.

This paper represents work done in part under contract with the Chemical Corps, U. S. Army, Washington 25, D. C.



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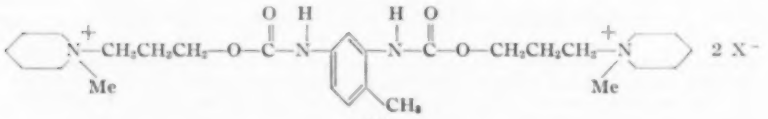
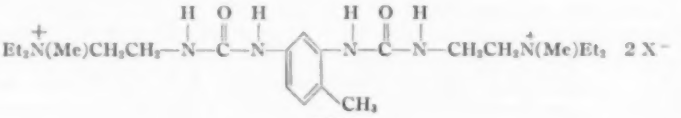


(V)

A series of carbamides, related to the carbamates of type I, can be prepared by reaction of the arylpolyisocyanates with N,N-disubstituted-ethylenediamines, etc., and one such carbamide, VI, was prepared. It is of interest to note that



(VI)

certain bis(2-tertiary-aminoalkyl)amides of succinic acid and its homologs have been found to potentiate the curariform action of succinylcholine and to antagonize that of *d*-tubocurarine chloride, while the dimethiodides were inactive (6).

EXPERIMENTAL

Arylpolycarbamates.—A solution of 0.1 mole of 1-methylphenylene-2,4-diisocyanate[1] in 40 ml. of dry toluene was added dropwise with stirring to a solution of 0.2 mole of an amino alcohol in 50 ml. of dry toluene during about thirty minutes. When addition was complete, the dropping funnel was rinsed with 10 ml. of toluene and the mixture was stirred at room temperature until evolution of heat ceased. The reaction was completed by heating under reflux for about five hours. Ligroin was added to the cooled reaction mixture to precipitate an oil which usua ly crystallized on rubbing with more ligroin. The crystalline or amorphous solid thus obtained was washed with ether and recrystallized when possible from ethanol or acetone. Yi lds of the arylpolycarbamates before recrystallization were 90 per cent or better. The arylpolycarbamates made in this way together with melting points and analytical data for the crystalline products appear in Table I.

By use of *p,p'*-diisocyanatodiphenylmethane in place of the 1-methylphenylene-2,4-diisocyanate in the above procedure together with 2-dimethylaminoethanol, an amorphous product was obtained and, from this, a crystalline methiodide was prepared as indicated below. When *p,p',p''*-triisocyanatotriphenylmethane was employed together with 2-dimethylaminoethanol, an amorphous product was obtained, and no crystalline salt could be produced from it.

Arylpolycarbamate Hydrochlorides.—The hydrochlorides were generally prepared in the usual way by treating the arylpolycarbamate with a solution of dry hydrogen chloride in absolute ethanol until the mixture gave an acid reaction on moist congo red paper.

It was also possible to prepare the hydrochlorides by reaction of the arylpolyisocyanate with the hydrochloride of the amino alcohol as illustrated in the following example. A solution of 8.7 Gm. (0.05 mole) of 1-methylphenylene-2,4-diisocyanate in 20 ml. of dry chloroform was added dropwise with stirring during thirty minutes to a solution of 18 Gm. (0.1 mole) of 3-(1-piperidyl)-1-propanol hydrochloride, m. p. 103–105°, reported m. p. 102–104° (7). The reaction was completed by refluxing gently for one hour. The mixture crystallized on standing and the product was recrystallized from a mixture of absolute ethanol and ether.

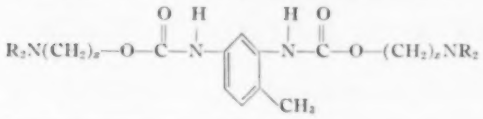
The hydrochlorides of the arylpolycarbamates are listed together with melting points and analytical data in Table I.

Methiodides of the Arylpolycarbamates.—*Method I.*—A mixture of 0.02 mole of the arylpolycarbamate and methyl iodide (0.05 mole for a dicarbamate, 0.075 mole for the tricarbamate) in methanol, ethanol, dilute ethanol or methyl ethyl ketone, was refluxed for about two hours. The choice of solvent depended on the solubility of the methiodide as determined in preliminary tests. The crystalline material which separated from the reaction mixture on cooling, was recrystallized from water, methanol, ethanol or a mixture of ethanol and ether. Yields of crude product, before recrystallization, were practically quantitative.

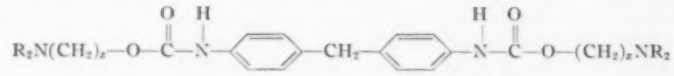
Method II.—A second procedure, involving, the reaction of the arylpolyisocyanate with the methiodide of the amino alcohol, was also employed. This procedure is illustrated by the following example. A solution of 0.87 Gm. (0.005 mole) of 1-methylphenylene-2,4-diisocyanate in 10 ml. of dry chloroform was added to a solution of 2.88 Gm. (0.01 mole) of 3-(1-piperidyl)-1-propanol methiodide, m. p. 133°, reported m. p. 133° (8), in 10 ml. of warm

[1] The authors acknowledge with appreciation the supply of arylpolyisocyanates by the Monsanto Chemical Company.

TABLE I.—ARYLPOLYCARBAMATES AND THEIR SALTS



$R_2N(CH_2)_x-$	m. p.	Formula	Anal. % N Calcd.	Found
2-Dimethylaminoethyl-(I)	116.5–117°	$C_{17}H_{28}N_4O_4$	15.90	15.36
I-Dihydrochloride	94–96°	$C_{17}H_{30}Cl_2N_4O_4 \cdot 3\ H_2O$	11.69	11.64
I-Dimethiodide	198–199°	$C_{19}H_{34}I_2N_4O_4 \cdot 2\ H_2O$	8.33	8.26
2-(1-Piperidyl)-ethyl-(II)	124.5–125.5°	$C_{23}H_{36}N_4O_4$	12.95	12.72
II-Dihydrochloride	135–137°	$C_{23}H_{38}Cl_2N_4O_4$	11.03	10.75
II-Dimethiodide	225–226°	$C_{25}H_{42}I_2N_4O_4$	7.84	7.81
2-(4-Morpholinyl)-ethyl-(III)	129–131°	$C_{21}H_{32}N_4O_6$	12.83	13.02
III-Dihydrochloride	142–144°	$C_{21}H_{34}Cl_2N_4O_6$	10.91	10.67
III-Dimethiodide	219° decompn.	$C_{23}H_{38}I_2N_4O_6$	7.77	7.68
3-(1-Piperidyl)-propyl-(IV)	amorphous			
IV-Dihydrochloride	102–104°	$C_{25}H_{42}Cl_2N_4O_4$	10.43	10.41
IV-Dimethiodide	120–122°	$C_{27}H_{46}I_2N_4O_4$	7.52	7.43
3-(4-Morpholinyl)-propyl-(V)	amorphous			
V-Dimethiodide	118–120°	$C_{25}H_{47}I_2N_4O_6$	7.49	7.30



2-Dimethylaminoethyl-(VI)	amorphous			
VI-Dimethiodide	177–179°	$C_{25}H_{38}I_2N_4O_4$	7.87	7.77

chloroform; and the mixture was refluxed for four hours. The solid which separated from the reaction mixture on cooling and standing, was recrystallized from a mixture of ethanol and ether. A yield of 83% of pure (recrystallized) product was obtained by this procedure.

The methiodides prepared as above directed are listed in Table I together with melting points and analytical data.

N,N′-Bis(2-diethylaminoethylcarbonyl)-toluene-2,4-diamine Dimethiodide.—A solution of 17.4 Gm. (0.1 mole) of 1-methylphenylene-2,4-diisocyanate in 50 ml. of dry toluene was added in portions with stirring and cooling to a solution of 25.6 Gm. (0.22 mole) of N,N-diethyl-ethylenediamine in 50 ml. of toluene. The reaction mixture was cooled as long as heat was evolved and the mixture was then stirred at room temperature for six hours. An amorphous semisolid separated and could not be crystallized. The reaction mixture was treated with 31.2 Gm. (0.22 mole) of methyl iodide and stirred at room temperature for five hours. The solid which separated from the reaction mixture was recrystallized repeatedly from mixtures of acetone and alcohol; white crystals, m. p. 105–108°.

Anal.—Calcd. for $C_{23}H_{44}I_2N_6O_2$: N, 12.17. Found N, 11.94.

SUMMARY

1. A series of arylpolycarbamates and the corresponding hydrochlorides and methiodides has been prepared because of the structural features which the methiodides have in common with such neuromuscular-blocking agents as succinyl choline and hexamethylenebis(carbamoylcholine).

2. The preparation of the methiodide of one number of a parallel series of carbamides is also described.

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Antispasmodic and Related Pharmacological Actions of Some γ-Arylpropylamines*

By DUANE G. WENZEL† and GILBERT H. EMICK

Seventeen γ-arylpropylamines were tested pharmacologically for their antispasmodic and other related actions. Simple methods for the *in vivo* evaluation of antiperistaltic activity and for anti-Parkinsonism activity are presented.

A SERIES OF γ-arylpropylamines synthesized by Burckhalter and Johnson (1, 2) were made available to us as the hydrochlorides for pharmacological testing. These compounds resemble several classes of drugs in having an amine group separated, in this case, by three carbons from aryl or other space-occupying groups. Most of the compounds fall into one of two subgroups: the first with an α-substituent and γ-diphenyl substitution and the second having both a phenyl and an ester group in the γ-position.

In this paper these compounds are reported for their antispasmodic and related activities. Studies of their toxicity, local anesthetic and antihistaminic effects will follow in a later publication. A number of commercial antispasmodics have been included in the testing in order to give a broader basis for comparison.

EXPERIMENTAL

Spasmolytic Action.—*Inhibition of Rat Ileal Sections.*—A modification of the apparatus and method of Picchioni and Edwards (3) was used to test the effectiveness of the compounds for reducing spasms of the circular muscles of the rat ileum. Black-hooded rats were used.

The spasmogen was added to the bath containing the muscle in Locke-Ringer's Solution U. S. P. XIV to produce the desired concentration (acetylcholine, $1:10^5$ or barium chloride, $1:10^4$). Activity was recorded kymographically with a tambour and two minutes after the addition of the spasmogen, when the contractions were uniform, the test drug was added. The minimal effective antispasmodic concentration was determined for each compound on not less than four ileal segments per concentration of compound. A positive response was considered to be a 75% reduction in the amplitude of contraction or spastic level. An active compound was one that gave a positive response in three out of four segments.

A summary of the results of the antispasmodic testing is shown in Table I. Four γ-arylpropylamines were twice as potent as papaverine in musculotropic antispasmodic activity. These were K95, K97, K239, and K269. K241 was five times as active against barium spasms as papaverine, equal to Pavatrine®, and one-half as active as Panparnit®. There are few if any structural similarities which set these γ-arylpropylamines apart from the others. One point worthy of note is the fact that compounds K241 > K239 ≃ K97 > K238 arranged in the order of decreasing musculotropic antispasmodic activity may be similarly arrayed with regard to the amount of substitution on the amine. On the whole, the γ-arylpropylamines tested were considerably less potent as neurotropic antispasmodics than most of the drugs used as standards. It is probable, however, that activity against acetylcholine spasms could have been enhanced by the use of the quaternary salts rather than the hydrochlorides (4). Antispasmodic activity, both musculotropic and neurotropic, was generally less with an ester group on the γ-position than when this position was occupied by another phenyl group.

Inhibition of Mouse Peristalsis.—The method used was derived in part from that of Fish (5). Male albino mice weighing from 18 to 25 Gm. were fasted for seventeen hours with water given *ad libitum*. During the eighteenth hour they were allowed free access to food. Ten animals were used per compound tested. One hour after having started to feed, each animal was orally administered 0.4 ml. of a 0.4% suspension of sudan III in 5% acacia and placed in a 600-ml. beaker. Ten minutes after the administration of the sudan III, the test compound or antispasmodic was given i. p. All compounds were administered in doses of 32 mg./Kg., except atropine which was given in a dose of 1 mg./Kg. Twenty minutes following the sudan III and every twenty minutes thereafter until the experiment was concluded, carbachol was injected i. p. in a dose of 0.2 mg./Kg. A control group was used to which carbachol but no antispasmodic was administered. The end point of the test was the detection of sudan III in the feces. This was accomplished by adding chloroform to the feces on a spot plate to bring out the color. Time was recorded from administration of the dye to its detection in the feces.

The mean times for the passage of sudan III are given in Table I. Although no direct comparisons can be made between the results of this test and the preceding one on the rat ileum, it appears that those γ-arylpropylamines having the greatest activity in the mouse peristalsis test were those that were active as both musculotropic and neurotropic antispasmodics.

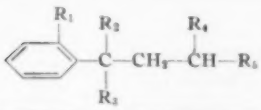
* Received August 6, 1955, from the School of Pharmacy, University of Kansas, Lawrence.

Abstracted from a portion of a thesis submitted by Gilbert H. Emick to the Graduate School of the University of Kansas in partial fulfillment of the requirements for the degree of Master of Science.

The authors wish to thank the Ciba Pharmaceutical Products Inc., for supplying Antrenyl®, Ba-10870 [3-phenyl-3-(β-diethylaminoethyl)-2,6-dioxopiperidine HCl] and Trasentine®; the Lederle Laboratories for Artane®; G. D. Searle and Company for Banthine®, Pavatrine® and Pro-banthine®; and Geigy Pharmaceuticals for Panparnit®.

† Associate Professor of Pharmacology, School of Pharmacy, University of Kansas.

TABLE I.—ANTISPASMODIC ACTIVITY



No.	R_1	R_2	R_3	R_4	R_5	Spasmolytic Activity Rat Ileal Sections[a] Barium	Acetylcholine	Mouse-Dye Defecation Time in Minutes ± S. E.[b]
K40	Hydrogen	Ethyl	Hydroxyl	Hydrogen	Dimethylamino	1:250,000	1:250,000	57 ± 2.1
K51	Hydrogen	Ethyl	Acetoxy	Hydrogen	Dimethylamino	1:100,000	1:250,000	68 ± 5.6
K57	Hydrogen	Ethyl	Propionoxy	Hydrogen	Dimethylamino	1:100,000	1:50,000	85 ± 3.8
K79	Hydrogen	Hydrogen	Propionoxy	Hydrogen	Piperidyl	1:500,000	1:500,000	109 ± 7.0
K80	Hydrogen	Hydrogen	Acetoxy	Hydrogen	Piperidyl	1:100,000	1:1,000,000	104 ± 5.8
K81	Hydrogen	Hydrogen	Propionoxy	Hydrogen	Morpholinyl	1:50,000	1:100,000	66 ± 12.8
K82	Hydrogen	Hydrogen	Acetoxy	Hydrogen	Morpholinyl	1:10,000	1:10,000	61 ± 1.9
K83	Methyl	Ethyl	Propionoxy	Hydrogen	Dimethylamino	1:500,000	1:500,000	106 ± 5.0
K84	Methyl	Ethyl	Acetoxy	Hydrogen	Dimethylamino	1:100,000	1:500,000	68 ± 6.0
K95	Hydrogen	Hydrogen	Hydrogen	Phenyl	Dimethylamino	1:1,000,000	1:250,000	95 ± 6.3
K97	Hydrogen	Hydrogen	Phenyl	Methyl	Methylamino	1:1,000,000	1:2,500,000	94 ± 7.9
K98	Hydrogen	Hydrogen	Phenyl	Phenyl	Dimethylamino	1:500,000	1:1,000,000	144 ± 15.3
K238	Hydrogen	Hydrogen	Phenyl	Methyl	Methylamino	1:250,000	1:1,000,000	178 ± 10.2
K239	Hydrogen	Hydrogen	Phenyl	Methyl	Dimethylamino	1:1,000,000	1:1,000,000	178 ± 5.6
K241	Hydrogen	Hydrogen	Phenyl	Methyl	Piperidyl	1:2,500,000	1:2,500,000	158 ± 6.1
K246	Hydrogen	Hydrogen	Phenyl	Methyl	Pyrrolidyl	1:500,000	1:2,500,000	89 ± 5.5
K269	Hydrogen	Propyl	Phenyl	Methyl	Dimethylamino	1:1,000,000	1:2,500,000	102 ± 8.1
Control (carbachol)						.....	.....	84 ± 4.0
Papaverine						1:500,000	.....	.....
Atropine						.....	1:100,000,000	142 ± 4.3
Antrenyl						1:50,000	1:100,000,000	440 ± 16.0
Artane						1:1,000,000	1:25,000,000	206 ± 10.2
BA-10870						1:10,000	1:2,500,000	314 ± 11.1
Banthine						1:100,000	1:2,500,000	330 ± 32.1
Panparnit						1:5,000,000	1:5,000,000	186 ± 15.4
Pavatrine						1:2,500,000	1:5,000,000	131 ± 4.2
Probanthine						1:500,000	1:250,000,000	407 ± 14.6
Trasentine						1:500,000	1:5,000,000	122 ± 8.6

[a] Effective concentration to reduce acetylcholine ($1:10^6$) or barium ($1:10^4$) spasms 75% in 3 out of 4 sections.
[b] Dose of all compounds 32 mg./Kg., except atropine 1 mg./Kg.

Mydriasis.—The method of testing was patterned after that of Pulewka (6). Male albino mice weighing from 18 to 25 Gm. were used in groups of five for each compound tested and the control. The compounds were injected i. p. and the pupillary size determined at periods of fifteen minutes, thirty minutes, one hour, two hours, and four hours. The γ-arylpropylamines and the commercial antispasmodics, except atropine, were first screened at a dose of 20 mg./Kg. Those compounds which increased the diameter at any of the times tested to over 15 units (33 units = 1 mm.) were then administered in decreasing dosage dividing by a factor of 2 until the diameter of the pupil was no more than 15 units or the dose was 2.5 mg./Kg. The observation of the pupil was performed by gently grasping the mouse by the head and placing the left eye under a stereoscopic microscope with a calibrated eyepiece. The light source was a 100-watt monowave bulb in a Burton lamp placed eight inches from the eye. The original method recommended the use of mice from 15 to 20 Gm. in weight and administering the same dose to all animals. While Pulewka (6) and later workers (7) using his method observed that heavier mice were less sensitive to mydriatics, we found any difference attributable to weight to be minimal providing the drug was administered on a weight basis and if a large weight range was avoided.

Table II shows the γ-arylpropylamines to be either relatively poor mydriatics or devoid of this activity at 20 mg./Kg. The results obtained with the standard antispasmodics more closely paralleled those of the mouse peristalsis test than the neurotropic activity on the rat ileum. Figure 1 illustrates the duration of mydriasis for the two most active γ-arylpropylamines and three typical standards.

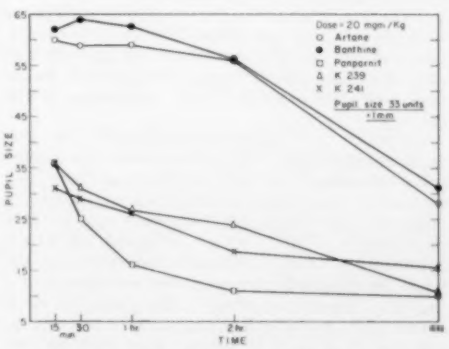


Fig. 1.—Duration of mydriatic effect.

TABLE II.—MYDRIATIC ACTIVITY IN MICE

Compound	Lowest Active Dose Tested[a]	Mean Maximum Pupil Diameter at this dose	Duration of Dilatation in Minutes[b]
K40	..	..	..
K51	..	..	..
K57	..	..	..
K79	..	..	..
K80	..	..	..
K81	..	..	..
K82	..	..	..
K83	..	..	..
K84	..	..	..
K95	..	..	..
K97	..	..	..
K98	..	..	..
K238	..	..	..
K239	5	17	15
K241	2.5	15	15
K246	10	16	15
K269	..	..	..
Atropine	0.1	19	120
Antrenyl	2.5	72	>240
Artane	2.5	31	15
BA-10870	2.5	55	20
Banthine	2.5	53	60
Panparnit	10	20	15
Pavatrine	20	21	15
Probanthine	2.5	61	120
Trasentine	20	16	15

[a] The lowest dose causing dilatation at any of the times tested. The lowest dose tested was 2.5 mg./Kg. except for atropine which was given as low as 0.025 mg./Kg. The criterion of a dilated pupil was a diameter >15 units (33 units = 1 mm.). Any dose causing such dilatation was considered an active dose. Compounds causing a dilatation of >15 units are indicated by a dash.

[b] Last time interval examined at which pupil size was >15 units.

Antisialogogue Action.—Male albino rabbits weighing from 2 to 3.7 Kg. were anesthetized with 27 mg./Kg. of sodium pentobarbital i. p. Four animals were used for each drug tested. All compounds were administered i. p. in a dose of 25 mg./Kg. except atropine which was given in a dose of 0.15 mg./Kg. These were immediately followed by 5 mg./Kg. of pilocarpine *via* the same route. Saliva was collected for a period of two hours from the administration of the sialogogue.

The mean volume of saliva secreted by each group over the two-hour test period is given in Table III. Most of the γ-arylpropylamines are ineffective as antisialogogues and the results are generally in accord with the other tests for parasympatholytic activity.

Inhibition of Chromodacryorrhea.—This test measures the ability of drugs to inhibit the methacoline-stimulated red-colored tears of rats (8). Groups of four black-hooded rats of either sex weighing from 250 to 300 Gm. were used for each dose tested. The γ-arylpropylamines were administered i. p. at a maximum dose of 64 mg./Kg. while the standard antispasmodics were tested at 1 mg./Kg. The dose of the latter was raised or lowered by a factor of 2 as necessary to produce the criterion of a positive effect. This criterion was inhibition of the red-colored tears in at least 50%

TABLE III.—ANTISIALOGOGUE ACTION IN RABBITS[a]

Compound[b]	Mean Two-Hour Secretion of Saliva in ml. ± S. E.
Control	68 ± 5.0
K40	80 ± 8.3
K51	95 ± 4.2
K57	67 ± 4.2
K79	70 ± 5.2
K80	44 ± 2.3
K81	88 ± 9.4
K82	84 ± 6.5
K83	70 ± 9.8
K84	96 ± 9.4
K95	.....[c]
K97	72 ± 3.8
K98	78 ± 16.4
K238	58 ± 6.0
K239	14 ± 3.0
K241	27 ± 1.8
K246	13 ± 4.3
K269	68 ± 2.7
Atropine	5 ± 1.1
Antrenyl	0 ± 0
Artane	1 ± 0.4
Ba-10870	0 ± 0
Banthine	0 ± 0
Panparnit	4 ± 1.6
Pavatrine	28 ± 10.2
Probanthine	0 ± 0
Trasentine	44 ± 6.4

[a] Rabbits anesthetized with sodium pentobarbital, 27 mg./Kg. Salivation induced by pilocarpine, 5 mg./Kg. i. p.

[b] All compounds administered in a dose of 25 mg./Kg. except atropine, 0.15 mg./Kg.

[c] Toxic at 25 mg./Kg.

TABLE IV.—INHIBITION OF CHROMODACRYORRHEA[a]

Compound	Dose in mg./Kg.	Number Protected / Number Tested
K40	64	0/4
K51	64	0/4
K57	64	0/4
K79	64	0/4
K80	64	0/4
K81	64	0/4
K82	64	0/4
K83	64	0/4
K84	64	0/4
K95	64	0/4
K97	64	0/4
K98	64	0/4
K238	64	0/4
K239	64	0/4
K241	64	0/4
K246	64	0/4
K269	64	0/4
Atropine	4	3/4
	2	1/4
Antrenyl	0.063	3/5
	0.031	0/4
Artane	64	2/4
BA-10870	2	5/5
	1	1/4
Banthine	0.5	3/5
	0.25	1/4
Panparnit	64	2/4
Pavatrine	64	0/4
Probanthine	0.125	5/5
	0.063	0/5
Trasentine	64	0/4

[a] Chromodacryorrhea was induced in rats by methacholine chloride, 10 mg./Kg. i. p.

of the animals at a particular dose. Fifteen minutes following the administration of the compound to be tested, methacholine chloride, 10 mg./Kg., was injected i. p. In an unprotected rat the red-colored tears were seen within a minute. The response was checked in doubtful cases by wiping the eye with absorbent cotton. As shown in Table IV none of the γ-arylpropylamines gave any evidence of activity at the 64 mg./Kg. dose tested. Obviously this procedure is not as sensitive a measure of antimuscarinic activity as the salivation test.

Nicotine Tremor Test.—Bovet and Longo (9) have described a method in which compounds potentially effective for Parkinsonism may be tested by their ability to reduce nicotine tremors in the rabbit. Essentially this procedure involves measurement of the reduction of leg tremor. It was modified in the present study by substituting rats for rabbits and by observing protection against the typical nicotine syndrome *in toto*. The symptom complex from the i. p. administration of 3 mg./Kg. of nicotine consists of: hard forced breathing with wheezing, body rigid and rocking from side to side, and the front legs and toes extended with an intermittent "dog-paddling" motion. Black-hooded rats of either sex weighing from 200 to 300 Gm. were used in groups of four for each drug tested. All compounds were administered i. p. in a dose of 75 mg./Kg unless toxicity was observed and then the dose was lowered to 50 mg./Kg. The test compound was followed in fifteen minutes by the challenging dose of nicotine. All compounds which abolished the nicotine syndrome as described were retested at lower doses using a factor of 1.5.

TABLE V.—NICOTINE TREMOR TEST[a]

Compound	Dose in mg./Kg.	Number Protected / Number Tested
K40	50	0/4
K51	50	0/4
K57	75	4/4
	50	1/4
K79	75	1/4
K80	50	6/7
	33	0/4
K81	50	0/4
K82	50	0/4
K83	50	0/4
K84	50	0/4
K95	50	0/4
K97	50	0/4
K98	50	0/4
K238	50	0/4
K239	50	0/4
K241	50	4/4
	33	2/4
K246	50	0/4
K269	50	0/4
Atropine	75	0/4
Antrenyl	75	0/4
Artane	75	0/4
BA-10870	75	0/4
Banthine	50	0/4
Panparnit	75	4/4
	50	2/4
	33	1/4
Pavatrine	75	0/4
Probanthine	75	0/4
Trasentine	75	3/4
	50	1/4

[a] Nicotine tremor of rats was induced by 3 mg./Kg. of nicotine i. p.

From the figures in Table V it may be concluded that the results with the standards are not entirely in accord with clinical reports of their activity. Artane® and atropine, for example, are widely used for Parkinson's disease yet showed up poorly in this test. With this observed lack of correlation it is not possible to definitely conclude that any of the γ-arylpropylamines would be active in Parkinsonism. It would be desirable, however, to clinically test those γ-arylpropylamines that prevented the nicotine syndrome.

DISCUSSION

As previously indicated, most of the compounds presented in this paper fall into one of two categories; they are either γ-diphenylpropylamines or γ-acetoxy- or propionoxy-γ-phenylpropylamines. While the ester linkage has been shown by others to be nonessential for anticholinergic or antispasmodic activity (10, 11, 12), it is found in the most potent cholinergic blocking agents (13). The esters studied in this work, however, are generally less active than the corresponding γ-diphenylpropylamines. One explanation is that the ester group is constituted as one of the terminal wings of the umbrella structure in these compounds rather than as a linkage interposed between the space-occupying groups and the amine nitrogen. It should be noted that the difference in activity may not be solely due to an ester *versus* a phenyl group in the γ-position as the γ-diphenyl compounds are different in having substitution on the α-position. This configuration may inhibit deamination of the compounds in the body as with the sympathomimetic amines. To resolve this problem would require the testing of two series of compounds which were identical except for the presence or absence of an α-substituent.

On the whole, none of the γ-arylpropylamines tested were exceptional as anticholinergic agents although compounds K97, K241, and K269 had good neurotropic and musculotropic antispasmodic activity. The possible activity against Parkinsonism of K57, K80, and K241, coupled with a relative lack of anticholinergic side-effects, may warrant further study of these compounds.

SUMMARY

1. Seventeen γ-arylpropylamine hydrochlorides and nine commercial antispasmodics as standards were tested for their antispasmodic and related activities.

2. Several test compounds demonstrated good neurotropic and musculotropic antispasmodic activity with relatively few side-effects.

3. Some of the γ-arylpropylamines were effective in preventing nicotine stimulation in rats and may be of value in Parkinsonism.

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The Fluorometric Assay for Chlortetracycline HCl and the Determination of Tetracycline HCl in Chlortetracycline HCl*

By FORTUNATO S. CHICCARELLI, PAUL VAN GIESON, and MASON H. WOOLFORD, Jr.

A procedure is described for the determination of chlortetracycline and for small quantities of tetracycline present in chlortetracycline. In a basic aqueous medium chlortetracycline degrades to isochlortetracycline which gives a blue fluorescence quantitatively measurable in the presence of small amounts of tetracycline. Tetracycline in an aqueous basic medium develops a yellow color which is quantitatively measurable in the presence of chlortetracycline.

CHLORTETRACYCLINE,[1] a yellow crystalline antibiotic, is obtained from *Streptomyces aureofaciens* (1). Since the introduction of chlortetracycline in 1948 the control assay has been based on the characteristic blue fluorescence of isochlortetracycline made by treating dilute solutions of chlortetracycline with strong base. Other methods of assay, fluorometric (2) and colorimetric (3–5), have been proposed but do not give the specificity or desired accuracy of the control assay currently in use by the Quality Control Section of Lederle Laboratories.

In addition to a method for chlortetracycline, there is also a need for a sensitive method for the determination of low concentrations of tetracycline[2] in chlortetracycline. In a basic aqueous medium, tetracycline develops a yellow color which is quantitatively measurable.

EXPERIMENTAL

A blue fluorescence which develops under basic conditions is characteristic for dilute chlortetracycline solutions. Tetracycline under the same conditions does not have this property.

The wavelength of maximum intensity of fluorescence is 428 mμ excited by essentially 365 mμ radiation through a Corning glass filter (No. 5840, 8 mm. thick). A phototube filter combination of Corning No. 5113 (2 mm. thick) and Corning No. 3389 (2 mm. thick) will provide the maximum transmission at this wavelength (6).

For increased accuracy, as with most fluorometric assays, a standard is determined with each sample of chlortetracycline. This eliminates variables inherent in the fluorometer, which at times make a concentration curve unreliable. However it has been determined that a concentration curve from zero to 3.5 μg./ml. is nearly linear under the conditions of the assay (Table I).

TABLE I

Concentration	Potentiometer Readings
1.0 μg./ml.	49.0
2.0 μg./ml.	97.0
3.0 μg./ml.	142.0
3.5 μg./ml.	164.0
4.0 μg./ml.	184.0
5.0 μg./ml.	215.0
6.0 μg./ml.	242.0
7.0 μg./ml.	267.0

* Received December 29, 1955, from Lederle Laboratories Division, American Cyanamid Co., Pearl River, N. Y.
The authors wish to acknowledge with gratitude the kind help of Dr. C. W. Waller of the Research Division toward the completion of this work, and to Mr. C. Hasse of the International Department for numerous suggestions.

[1] The trademark of Lederle Laboratories Division, American Cyanamid Company for chlortetracycline is Aureomycin.®

[2] The trademark of Lederle Laboratories Division, American Cyanamid Company for tetracycline is Achromycin.®

Under these conditions, quenching has an effect when solutions at a higher concentration than 3.5 μg./ml. are assayed. In all control work the assay is performed using standards and samples which when diluted will give a concentration of about 3.0 μg./ml. Sodium hydroxide is added to give a final concentration of 0.25 *N*.

The effect of time on the conversion to and the stability of isochlortetracycline is shown in Table II.

TABLE II

Time in Minutes	Potentiometer Readings
1.0	113.0
2.5	133.0
4.0	135.0
7.0	136.0
10.0	136.0
15.0	136.0
18.0	136.0
21.0	136.0
27.0	135.0
33.0	134.0

Fifteen minutes was chosen between the addition of sodium hydroxide and the actual fluorometric measurement in order to allow time for the instrument preparation.

The Klett Photofluorometer (Model 2070) is used at the present time for the fluorescence measurement. Since the fluorescence of isochlortetracycline fades on exposure to ultraviolet light, the fluorescence is determined thirty seconds after the opening of the shutter by measuring with the potentiometer the deflection right or left of zero on the mirror galvanometer.

Since there is also a need for a sensitive method for determining low concentrations of tetracycline in chlortetracycline, a search was made for a chemical reaction given by tetracycline and not by chlortetracycline. As the two substances are very similar it became apparent that they would both give the same kind of chemical reaction with most reagents.

In two instances, however, definite differences were noted. They are: (*a*) H_2SO_4 (concentrated): tetracycline, deep violet; chlortetracycline, deep blue (changing to green and then brown). (*b*) NaOH(0.25 *N*): tetracycline, definite yellow color without fluorescence having an absorption maximum at 380 mμ; chlortetracycline, colorless with fluorescence having an absorption maximum at 345 mμ.

The differences in absorbance in concentrated sulfuric acid were too slight to be resolved. However, under basic conditions, tetracycline develops a maximum absorbance at 380 mμ while that of chlortetracycline is at 345 mμ. If tetracycline is present in chlortetracycline, under basic conditions, it will be shown by a shelf developing at 380 mμ in the absorption spectrum of chlortetracycline. The height developed by this shelf is proportional to the amount of tetracycline present. In order to make use of this shelf formation, the chlortetracycline must have a concentration of at least 200 μg./ml. in 0.25 *N* NaOH.

The assay was carried out by transferring 50 ml. of a 400 μg./ml. chlortetracycline solution to a 100-ml. volumetric flask. After adding enough water to make the total volume about 90 ml., two ml. of 10% $NaHSO_3$ was added to prevent oxidation. The final concentration of 0.25 *N* NaOH was produced by adding 5 ml. of 5 *N* NaOH and diluting to 100 ml. with water. In preparing solutions for a standard curve, part of the standard chlortetracycline solution was withdrawn from the original 50 ml. and the desired amount of tetracycline was added to give concentrations of tetracycline up to 6%. The total concentration of antibiotic was always 200 μg./ml.

Table III shows the effect of time on the color development. The temperature of the solution was 25°.

TABLE III

Concentration (200 μg./ml. Total Antibiotic)	Absorbance at 380 mμ After Addition of NaOH — One Hour	Two Hours	Three Hours
A. 100% CTC[a]	0.365	0.350	0.353
B. 98% CTC + 2% TC[b]	0.503	0.467	0.470
C. 96% CTC + 4% TC	0.640	0.590	0.580
D. 94% CTC + 6% TC	0.780	0.720	0.690

[a] CTC—Chlortetracycline HCl. [b] TC—Tetracycline HCl.

Under these conditions time as a variable did not have an effect on the application of Beer's law within this range; however, time definitely affects the slope of the line. All subsequent determinations were made one hour after the addition of NaOH.

Table IV shows the effect of temperature on the color development. The absorbances were read one hour after the addition of NaOH.

TABLE IV

Concentration of Solution (200 μg./ml. Total Antibiotic)	Absorbance at 380 mμ — 25°	30°	37°
A. 100% CTC[a]	0.369	0.390	0.390
B. 98% CTC + 2% TC[b]	0.505	0.520	0.513
C. 96% CTC + 4% TC	0.645	0.640	0.625
D. 94% CTC + 6% TC	0.783	0.750	0.743

[a] CTC—Chlortetracycline HCl. [b] TC—Tetracycline HCl.

The temperature does not affect the application of Beer's law, however, the temperature has a definite effect on the slope of the line.

With all determinations it was decided to run a group of standard solutions along with the material being assayed. As a curve must be prepared with each sample in any case, room temperature was substituted in place of 25° to simplify the assay.

Assay of Chlortetracycline HCl

Apparatus.—The Klett Fluorometer, model 2070 with lamp filter Corning No. 5840 (8 mm. thick) and phototube filter Corning No. 5113 and No. 3389 (each 2 mm. thick).

Standard.—Weigh accurately exactly 100 milligrams of standard chlortetracycline HCl and transfer quantitatively to a liter volumetric flask. Add about 200 ml. of water, shake until solution is complete, and make to volume with water. This solution should be kept in an amber-colored flask and stored in a refrigerator. It must be prepared fresh

monthly. When needed, place 10–15 ml. in a small flask and allow the temperature of the solution to rise to room temperature.

Procedure.—Weigh accurately about 0.10 Gm. of sample into a liter flask. Add about 200 ml. of water and shake until dissolved. After solution is complete make to volume and mix. Pipet exactly 6 ml. of this solution and 6 ml. of the standard solution into 200-ml. volumetric flasks. To the standard and sample add about 180 ml. of water, and mix. Now add 10 ml. of 5 *N* NaOH, make to volume with water, and mix. Between fifteen and twenty minutes after the addition of NaOH, determine the fluorescence.

With the appropriate filters in the proper position, set the potentiometer at a reading of 75. Fill the rectangular cuvette with quinine sulfate solution (0.100 mg./100 ml. 0.1 *N* H_2SO_4) and standardize the instrument by adjusting the slit so that there is zero deflection on the galvanometer when the shutter is opened and the galvanometer knob is pushed to "H." The slit width must not be altered during any series of sample and standard readings. Rinse the cuvette well with water and twice with standard. Fill the cuvette with standard solution. Immediately after the shutter is opened and the galvanometer knob is pushed to "H," start the time measurement with a stop watch. Adjust the potentiometer so that the galvanometer is at zero in exactly thirty seconds. The sample should be measured in the same manner.

The per cent chlortetracycline is calculated by comparing the sample reading to the standard reading and multiplying by the dilution factor.

Determination of Tetracycline in Chlortetracycline

Reagents.—*Standard Chlortetracycline HCl.*—Weigh accurately exactly 200 mg. of standard and transfer to a 500-ml. volumetric flask. Add about 200 ml. of water and shake until dissolved. After solution is complete make to volume with water and mix.

Standard Tetracycline HCl.—Weigh exactly 100 mg. of standard tetracycline HCl and transfer quantitatively to a 500-ml. volumetric flask. Add about 200 ml. of water and dissolve the powder by shaking, then make to mark with water, and mix.

Procedure.—Weigh accurately exactly 200 mg. of sample and transfer to a 500-ml. volumetric flask. Add about 200 ml. of water and shake until dissolved. After solution is complete make to volume with water and mix. Transfer a 50-ml. aliquot to a 100-ml. volumetric flask.

Prepare the solution for the standard curve by transferring four separate 50-ml. aliquots of standard chlortetracycline HCl solution to four 100-ml. volumetric flasks. From one flask withdraw[1] exactly 1.0 ml. of the solution and add exactly 2.0 ml. of standard tetracycline HCl solution. This is the 2% tetracycline solution. From another flask withdraw 2.0 ml. of the solution and add 4.0 ml. of the standard tetracycline solution. This is the 4% tetracycline solution. From another flask withdraw 3.0 ml. of the solution and add 6.0 ml. of the standard tetracycline solution. This is the 6.0% tetracycline HCl solution. One flask is left unaltered. This is the 100% chlortetracycline HCl solution.

To each of the samples and standards add enough water to make a volume of about 90 ml.; then add 2 ml. of 10% $NaHSO_3$ and 5.0 ml. of 5 *N* NaOH; and make to volume with water, and mix. Allow to remain at room temperature for one hour. Read the absorbance on a suitable spectrophotometer against a water blank at 380 mμ.

Plot the per cent concentration against absorbance on arithmetical graph paper. Refer to this curve for the per cent tetracycline HCl in the sample.

SUMMARY

1. Chlortetracycline under basic conditions has a blue fluorescence. A control assay has been developed using this property.

2. Traces of tetracycline in chlortetracycline, under basic conditions, produce a shelf at 380 mμ in the spectrum of chlortetracycline, the height of which is dependent on the concentration of tetracycline.

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[1] Although the technique of withdrawal is not normally desirable, in the case of this assay, the reading which would be obtained at 380 mμ from the 0.1 or 0.2 ml. of chlortetracycline remaining in the pipet is negligible.

The Accumulation of Hordenine in the Seedlings of *Panicum miliaceum**

By G. E. DEMAREE and V. E. TYLER, Jr.

A quantitative paper chromatographic technique for the determination of hordenine by the maximum color density method is described. Application of this procedure to portions of proso seedlings during various stages of germination revealed that the principal site of accumulation of hordenine in that plant is in the shoots. The alkaloid content of these organs of both etiolated and light-grown seedlings was shown to increase from the third to the eighth day, as calculated on a per shoot basis. The presence of trace amounts of hordenine and uncombined tyramine in the roots and seeds of the plants during certain stages of germination was also noted. The significance of these findings and their relation to the pattern of hordenine accumulation in barley seedlings are discussed.

HORDENINE was first isolated from barley (*Hordeum vulgare* L.) by Leger in 1906 (1), and since then numerous investigators have studied its occurrence, distribution, and formation in that plant. Much of their work has been summarized by James (2) in a recent review. It has been established that the alkaloid does not occur in ungerminated barley seeds and its formation is restricted to the young roots of the plant. In these organs hordenine reaches a maximum concentration in about two weeks and then rapidly disappears. Although some of the earlier steps in the pathway are in dispute, it is generally agreed that hordenine is formed by two successive methylations of tyramine, the product accumulating in the roots of the barley seedlings.

Hordenine has also been shown to occur in other members of the *Gramineae*, and its presence was demonstrated in proso (*Panicum miliaceum* L.) seedlings in 1920 (3). Proso, nevertheless, seems to have been generally overlooked as a source of information on hordenine metabolism, and the present investigation was undertaken to determine the behavior of the alkaloid in this plant in comparison with barley.

Before proceeding with this phase of the study it seemed necessary to develop a sensitive, yet specific, method for the determination of hordenine in plant tissue extracts. Since the alkaloid is frequently accompanied in barley by tyramine and *N*-methyltyramine, an existing chromatographic procedure for the separation of these closely related substances (4) was adapted to quantitative measurement by developing a maximum color density method.

EXPERIMENTAL

Hordenine Standard Curve.—Commercial hordenine sulfate was utilized for the preparation of hordenine base, which after recrystallization from a mixture of benzene and petroleum ether exhibited a micro m. p., K. = 117–118°. Standard solutions were prepared from this base by dissolving sufficient quantities in alcohol so that the final solutions contained, 4, 8, 12, 16, and 24 μg. of hordenine in each 10 μl., respectively.

Ten-μl. volumes of the standard solutions were spotted in replicate on 1 × 22½ in. strips of Whatman No. 1 filter paper which had been buffered by dipping in McIlvaine's buffer at *p*H 8 and air dried. Ascending formation of the strips was carried out in a Chromatocab, Model A, employing *n*-butanol saturated with water as the wash liquid and water saturated with *n*-butanol as the external phase. Time of formation was twenty-four hours.

At the conclusion of this period the strips were air dried and the alkaloidal spots rendered visible by subjecting the strips to Pauly's reaction with sodium nitrite as described by Block, *et al.* (5). Treated in this way hordenine appeared as bright reddish-orange spots on the nearly white background of the paper. The method proved to be extremely sensitive, and quantities as small as 2 μg. could be detected readily.

A Photovolt Electronic Densitometer, Model 525, fitted with a 1 × 15 mm. slit was used to determine the maximum color density of the resultant spots. Employing the available filters for the instrument, which ranged from 420 to 630 mμ in 20–30 mμ intervals, it was found that the colored spots formed by hordenine exhibited a peak density near 465 mμ. A narrow-band gelatin filter of this wavelength was subsequently employed for all determinations. It was also determined experimentally that the density of the colored spots reached a maximum by the time the final reagent application had dried, and no appreciable change had occurred after an additional two hours. In the interest of uniformity all density readings were made as soon as possible after the strips had dried following application of the reagents.

Because of slight variations in the uniformity of the boundaries of the alkaloidal spots and variations in the density of the filter paper strips, a minimum of 22 strips was used to determine each point in the standard curve. Occasionally, for reasons which could not be ascertained, the alkaloidal spots appeared rather elongated, and strips exhibiting this type of formation were discarded. These strips

* Received November 19, 1955, from the Department of Pharmacognosy, University of Nebraska, College of Pharmacy, Lincoln.

could be distinguished readily from acceptable ones because in the former the alkaloid tended to concentrate along the edges of the paper. It is estimated that about 20% of the total strips formed were discarded because of this irregularity of spot formation. This did not complicate the assay procedure to any appreciable extent since the strips showing irregular spot formation were readily distinguishable, and a few extra strips could be added in order to obtain any desired minimum number. The relationship between maximum color density of the spots and hordenine concentration is shown in Table I.

TABLE I.—RELATIONSHIP OF MAXIMUM COLOR DENSITY AND HORDENINE CONCENTRATION ON FILTER PAPER STRIPS

Hordenine (μg.)	Density Scale Reading[a]	No. of Strips
4	[illegible] ± 0.02	22
8	0.21 ± 0.04	23
12	0.27 ± 0.04	26
16	0.32 ± 0.03	24
24	0.40 ± 0.04	22

[a] Expressed as the mean ± the standard deviation.

$$\text{Standard deviation} = \sqrt{\frac{\Sigma(X - \bar{X})^2}{N - 1}}$$

Analysis of Proso Seedlings.—Quantities of proso seed were germinated in moist air as described by Meyer and Anderson (6). Temperature during germination was controlled at 26 ± 2°, and one-half the seed was subjected to normal diurnal variations of light; the other half was germinated in continual darkness. One hundred seedlings from both the light-grown and the etiolated groups were harvested after two, three, four, six, and eight days of germination. A similar number of ungerminated seeds was also tested.

The shoots, roots, and seeds were separated, dried in an oven at 45° for twenty-four hours, and then in a desiccator over anhydrous calcium chloride at room temperature for an additional twenty-four hours. The dried parts were weighed and ground in a Wiley Laboratory Mill to a #20 powder. The powders thus obtained were reweighed, placed in 16 × 85 mm. test tubes together with 10 ml. of alcohol, the tubes stoppered and shaken on a reciprocal shaking machine for two hours. After centrifuging, the extracts were decanted and the residues washed with two successive 2-ml. portions of alcohol which were combined with the original extracts. Each extract was then concentrated in a hot water bath to a 1.0-ml. volume.

The application of Pauly's reaction as a qualitative test indicated that hordenine was completely extracted from the plant parts by the above technique. The use of the quantitative method previously described indicated that the short exposure to the heat of the water bath necessary to concentrate the extracts did not result in a detectable change in the total quantity of hordenine present. The concentrated extracts thus obtained were spotted on buffered filter paper strips of the type employed in preparing the hordenine standard curve. Spotting was carried out using replicate 10-μl. volumes allowing each volume to dry completely before adding the next. The total volume spotted was determined empirically so as to obtain maximum densities within the range of the prepared standard curve for hordenine. Formation and development of the strips were carried out as previously described. Spots were identified as hordenine or tyramine by their respective R_f values, but no spots corresponding to *N*-methyltyramine were observed on any of the strips from the various plant parts. The maximum densities of the hordenine spots were determined as indicated, and the amount of hordenine in the shoot extracts, the only parts containing measurable quantities, was calculated. These results are expressed both on a dry weight and per shoot basis in Table II.

TABLE II.—RESULTS OF HORDENINE DETERMINATION IN SHOOTS OF PROSO SEEDLINGS

Age of Sample Days	Light-Grown		Etiolated	
	Hordenine % Dry Wt.	Hordenine Per Shoot (μg.)	Hordenine % Dry Wt.	Hordenine Per Shoot (μg.)
2	....[a]	....[a]	....[a]	....[a]
3	0.89	4.1	0.51	2.2
4	0.72	4.5	0.36	1.8
6	0.73	6.4	0.49	3.6
8	0.71	7.1	0.39	4.8

[a] Not developed sufficiently to collect.

Neither hordenine nor uncombined tyramine was detected in the ungerminated seed. Hordenine and tyramine were both found to be present at all times in seeds separated from the light-grown plants although never in sufficient quantity to be determined quantitatively. Hordenine was present in seeds separated from the etiolated plants throughout the duration of the experiment, but tyramine could not be detected after four days. Again neither compound was present in measurable quantity.

Tyramine could not be detected in any root sample. Hordenine was present after four days in the light-grown roots and after six days in the roots of the etiolated plants, but never in a quantity which could be measured by the method employed.

Tyramine was present in shoot extracts of both light-grown and etiolated plants throughout the sixth day of germination, but could not be detected in either at the conclusion of the experiment on the eighth day.

DISCUSSION

The method described proved to be quite suitable for the quantitative determination of hordenine. It could no doubt be expanded to embrace tyramine and *N*-methyltyramine as well, thus providing a specific quantitative method for the determination of these three closely related substances.

Application of the procedure to extracts of proso seedlings revealed that the pattern of accumulation of hordenine in this plant is quite different from that known to occur in barley. In the shoots of both light-grown and etiolated proso seedlings hordenine is detectable on the

third day and continues to accumulate in increasing quantities, calculated on a per shoot basis for at least eight days. Although detectable quantities of hordenine were found in the roots and the seeds separated from these shoots, no measurable quantities of hordenine were found in those parts. It follows that the principal site of accumulation of hordenine in proso is the shoot as opposed to the root of barley seedlings. It should be emphasized that this observation is concerned only with the site of hordenine accumulation and not synthesis, since recent physiological evidence (7) indicates that the principal site of synthesis of most alkaloids is the root.

Evidence obtained in this experiment to substantiate the conversion of tyramine to hordenine is inconclusive. However, the fact that tyramine ultimately disappeared from the shoots of both light-grown and etiolated samples, as hordenine increased, may be considered as presumptive evidence of such a reaction.

The disappearance of tyramine from the seeds of etiolated plants after four days but its presence after eight days in the seeds of the light-grown plants suggest that the photosynthetic mechanism may influence the utilization of tyramine in the growing seedling.

Studies aimed at elucidating the role of the proso root in hordenine biosynthesis are now in progress in this laboratory.

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The Synthesis and Pharmacology of Some Polymethylene Bisonium Compounds*

By A. R. WILLIAMS†, JOHN HIDALGO, and I. F. HALVERSTADT

The sulfonium analogs of pentamethonium and hexamethonium and a series of S,S′-polymethylenebisthiuronium salts have been prepared. These compounds have been tested for hypotensive, spasmolytic, local anesthetic, and anticoagulant activity. They showed a variable hypotensive activity of short duration, a weak spasmolytic activity, and were found to be devoid of local anesthetic properties. Only S,S′-hexamethylenebis (thiuronium bromide) had any anticoagulant activity.

HEXAMETHONIUM and, more recently, pentapyrrolidinium bitartrate, have been shown to be potent ganglionic blocking agents in humans; however, their somewhat unpredictable absorption following oral administration and the numerous side effects which arise from their administration somewhat limit their use clinically for the control of hypertension. In an attempt to improve upon these compounds, we speculated that the replacement of the two charged centers with an atom other than N might change the properties of the compounds in a desirable way. Specifically, we hoped to decrease the toxicity, and perhaps increase the ratio of sympathetic to parasympathetic blockade, since the undesirable side effects of the ganglionic blockers (constipation, urinary retention, etc.) are due to parasympathetic inhibition. We were thus led to investigate the sulfur analogs of hexamethonium and pentamethonium. In addition, S,S′-hexamethylenebis(thiuronium bromide), obtained as an intermediate, and several homologous thiuronium compounds were tested.

Another series of hexamethonium analogs was planned, in which the ammonium groups were incorporated into a heterocyclic nucleus. A number of these compounds were being prepared when the extensive series of similar compounds prepared by Libman, Pain, and Slack (1) appeared in the literature, and work on this series was stopped. Two of the compounds in this series, which were not reported in the article by the British workers, are included in this paper.

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METHODS[1]

Chemistry

All melting points reported were determined on a Fisher-Johns microblock and are uncorrected.

General Procedure.—*S,S'-Polymethylenebis(1,3-dialkylthiuronium Bromide).*—The general procedure followed was that used by Walker (2). A mixture of 0.05 mole of polymethylene dibromide and 0.1 mole of the thiourea in 100 ml. of 95% ethanol was heated under reflux for four hours. The solvent was evaporated and the residue recrystallized from the solvents specified. The compounds prepared are listed in Table I.

The 1,3-dimethylthiourea was prepared from methyl isothiocyanate and aqueous methylamine according to the procedure of Hecht (3). An attempt to prepare the compound by the procedure used by Salkowski (4) resulted in the isolation of methylammonium N-methyldithiocarbamate (5), m. p. 116°, in nearly quantitative yield.

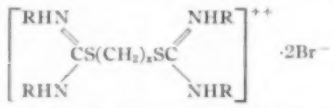
Anal.—Calcd. for $C_3H_{10}N_2S_2$: C, 26.1; H, 7.3; N, 20.3; S, 46.4. Found: C, 26.9; H, 7.0; N, 20.1; S, 46.2.

Hexamethylenebis (dimethylsulfonium Iodide).—Walker's (2) procedure for the preparation of pentamethylenebis(dimethylsulfonium bromide) was followed. The hexamethylenebis(thiuronium bromide) was prepared from 0.1 mole of 1,6-dibromohexane and 0.2 mole of thiourea, as described above. The crude bisthiuronium salt obtained by the evaporation of the solvent was dissolved in 240 ml. of 10% sodium hydroxide and was shaken with 31.2 Gm. (0.22 mole) of methyl iodide for two hours. The organic layer was removed and the aqueous phase was extracted with three 50-ml. portions of ether. After drying over magnesium sulfate, the combined organic extracts were distilled, yielding 13.5 Gm. (76%) of colorless 1,6-bis(methylthio)hexane, b. p. 150–152°/25 mm. A solution of this material and 26.0 Gm. (0.18 mole) of methyl iodide in 15 ml. of methanol was kept at 40–50° for twenty hours. The solution, after cooling, deposited 32 Gm. (91% yield) of pale yellow crystals, m. p. 140–144°. After two recrystallizations from methanol there was obtained 30.1 Gm. of colorless crystals, m. p. 143–146°.

Anal.—Calcd. for $C_{10}H_{24}I_2S_2$: S, 13.9. Found: S, 13.7.

Pentamethylenebis (dimethylsulfonium Iodide).—This compound, m. p. 179–181° [m. p. 182–183° was reported by Walker (2)], was prepared as above. The intermediate, 1,5-bis(methylthio)pentane, had b. p. 133–134°/28 mm.

TABLE I.—S,S'-POLYMETHYLENEBISTHIURONIUM COMPOUNDS



R	X	m. p.	Crystallization Solvent	Analysis—Calculated N	Analysis—Calculated Br	Analysis—Found N	Analysis—Found Br	Yield %	Mouse I.V. MLD mg./Kg.	Hypotensive[a] Activity in the Rat
H	6	203–205°[b]	Methanol	14.1	...	14.2	...	89	50	3/40, 3/40[c,d]
CH_3	6	137°	Ethanol	...	35.3	...	34.5	92	100	1/30
C_2H_5	6	119–120°	Methanol-acetone	11.0	...	10.8	...	78	25	4/30, 10/30[c,e]
i-C_3H_7	6	169–170°	Methanol-acetone	9.9	...	9.8	...	41	25	3/20, 7/30[c]
n-C_4H_9	6	90–93°	Acetone	9.0	...	8.8	...	74	16	5/40, 5/40
C_5H_5	2	189–190°	Ethanol	...	35.3	...	35.5	76	75–100	0/40

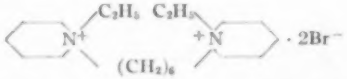
[a] Hypotensive activity expressed as time in minutes blood pressure remained lower than the starting pressure by 20 mm. Hg or more for 1 mg./Kg. dose of compound over time response to 1 mg./Kg. dose of pentapyrrolidinium bitartrate.
[b] Literature (11) reports m. p. 210–211°.
[c] Compounds had 1/50 to 1/100 the activity of atropine in antagonizing acetylcholine induced spasm on isolated rabbit ileum.
[d] Concentration of 0.5% prevents blood coagulation *in vitro*.
[e] Showed slight curare like activity.

An attempt was made to use the procedure that Bohme and Krause (6) used to prepare monosulfonium halides from dimethyl sulfide. A solution of 6.0 Gm. (0.0185 mole) of 1,5-diiodopentane and 5.0 Gm. (0.0805 mole) of dimethyl sulfide in 30 ml. of acetone and 1 ml. of water, was heated under reflux for eight and one-half hours and, on cooling in the ice chest, deposited 5.3 Gm. of yellow crystals, m. p. 180–185° (decompn.). After three recrystallizations from 95% ethanol and one from methanol, there was obtained 3.4 Gm. of colorless crystals which decomposed from 203–205° to volatile products without melting. This behavior on heating is in agreement with that reported for trimethylsulfonium iodide (7).

Anal.—Calcd. for $C_5H_{11}IS$: S, 15.7. Found: S, 15.2.

Although this product was not expected, there is some precedence for its formation (8).

1,1'-Hexamethylenebis(1-ethylpiperidinium Bromide).—



A solution of 12.2 Gm. (0.05 mole) of 1,6-dibromohexane and 16 Gm. (0.14 mole) of 1-ethylpiperidine in 40 ml. of 2-propanol was heated at 70° for five

[1] The microanalyses were run by either the Chemical Control Department of these Laboratories, or Microchemical Specialties Company, Berkeley, Calif.

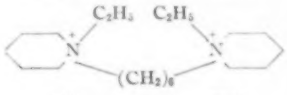
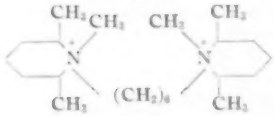
days. After chilling thoroughly there were obtained dense, tan crystals. After one recrystallization from 200 ml. of 2-propanol, there was obtained 19.1 Gm. (81% yield) of colorless crystals, m. p. 279–281°. A further recrystallization from 200 ml. of 2-propanol gave 17.8 Gm. (76% yield) of crystals, m. p. 284–285°.

Anal.—Calcd. for $C_{20}H_{42}Br_2N_2$: N, 6.0. Found: N, 6.1.

Pharmacology

The blood pressure of cats was recorded from the carotid artery through a mercury manometer. In rats the carotid artery was connected to an electromanometer and a "Twin Beam" Sanborn recorder. Pentapyrrolidinium bitartrate was used as the reference compound. Hypotensive activity was expressed as minutes at a blood pressure lower than the starting pressure by 20 mm. Hg or more. When more than one animal was studied, the order of administration of the compounds was reversed in the second animal. All compounds were administered intravenously.

TABLE II.—POLYMETHYLENE SULFONIUM AND PIPERIDINIUM COMPOUNDS

Structure	Mouse I.V. MLD mg./Kg.	Hypotensive Activity
$(CH_3)_2\overset{+}{S}-(CH_2)_5-\overset{+}{S}(CH_3)_2 \cdot 2I^-$	100	Rat: 1 mg./Kg. produced an erratic drop in blood pressure. Cat: Infusion of 5 mg./Kg./min. for ten minutes caused on fall in blood pressure.
$(CH_3)_2\overset{+}{S}-(CH_2)_6-\overset{+}{S}(CH_3)_2 \cdot 2I^-$	150	Cat: 10 mg./Kg. produced no change in the blood pressure.
 $\cdot 2Br^-$	5	Cat: 1 mg./Kg. produced drops of blood pressure of 30–40 mm. Hg lasting 6–10 minutes.
 $\cdot 2I^-$	3	Rat: 0.5 mg./Kg. produced drop of 0 to 75 mm. Hg for up to 15 minutes' duration. 1 mg./Kg. caused a profound and irreversible drop in blood pressure.

1,1′ - Hexamethylenebis(1 - methyl - 2,6 - meso-lupetidinium Iodide).—2,6-Lupetidine (*meso* form) was prepared according to the procedure of Marcuse and Wolffenstein (9). A solution of 0.17 mole of 2,6-lupetidine (prepared from 25 Gm. of the hydrochloride salt) and 18.2 Gm. (0.074 mole) of 1,6-dibromohexane in 100 ml. of 1-butanol was heated under reflux for forty-four hours. On cooling, there was obtained 22 Gm. of colorless crystals, m. p. > 300°. Concentration of the mother liquors yielded an additional 2 Gm. of colorless crystals. The 24 Gm. of crystals was converted to the free base which was recrystallized from aqueous methanol to give 8.5 Gm. of colorless crystals, m. p. 73–74°.

Anal.—Calcd. for $C_{20}H_{40}N_2$: C, 77.9; H, 13.1. Found: C, 76.8; H, 12.7.

A solution of 4 Gm. (0.013 mole) of 1,1′-hexamethylenebis(2,6-lupetidine) and 20 Gm. (0.14 mole) of methyl iodide in 25 ml. of methanol was heated under reflux for sixteen hours. To the warm solution was added 25 ml. of ether and, after cooling, there were obtained colorless crystals. After one recrystallization from 20 ml. of methanol and 20 ml. of ether there was obtained 4.1 Gm. of colorless crystals, m. p. 252°.

Anal.—Calcd. for $C_{22}H_{46}I_2N_2$: C, 44.6; H, 7.8. Found: C, 44.4; H, 7.6.

An attempt to prepare the diethiodide compound failed. When 1,1′-hexamethylenebis(2,6-lupetidine) and ethyl iodide were heated under reflux for seven hours a good yield of 1,1′-hexamethylenebis(2,6-lupetidine) dihydriodide, m. p. > 300° was obtained.

Anal.—Calcd. for $C_{20}H_{42}I_2N_2$: C, 42.6; H, 7.5. Found: C, 42.3; H, 7.8.

Spasmolytic activity was tested on isolated rabbit ileum suspended in aerated Tyrode's solution at 37°. The compound was first added to the bath in concentrations up to 1 mg./ml. to record the effect on the spontaneous activity of the muscle. The ability of the compound to relax the spasm induced by 0.01 to 0.04 μg./ml. of acetylcholine was tested on a separate muscle preparation.

Local anesthetic activity was determined in the eyes of rabbits by instilling 0.1 ml. of a 1% solution into the lachrymal sac and testing the cornea at one minute intervals by pressure with a blunt probe.

The anticoagulant activity[2] was tested by adding measured amounts of freshly drawn rabbit blood to tubes which contained aliquots of a solution of the compounds under study. Three tubes were used for each concentration and they were prepared and tested by the Lee-White procedure.

RESULTS

The hypotensor data for the S,S′-polymethylenebisthiuronium salts are summarized in Table I. The hypotensor data for the other compounds are summarized in Table II.

As may be seen, the sulfonium analogs were much less toxic than pentapyrrolidinium. They

[2] We should like to acknowledge the assistance of Alfred H. Fowell, of these Laboratories, for the anticoagulant studies.

were, however, rather unreliable hypotensors and even in large doses did not prevent the blood pressure rise caused by bilateral carotid occlusion in the cat.

The thiuronium compounds were also hypotensors but their duration of action was brief.

The piperidinium compounds were very toxic and did not seem to be sufficiently long acting.

A number of the thiuronium compounds, as noted under Table I, had a slight antispasmodic effect. All compounds were devoid of curare-like activity and local anesthetic activity.

Because of a report by Dirscherl and Weingarten (10) that S,S′-polymethylenebisthiuronium salts had anticoagulant properties, those of this series were tested *in vitro* for anticoagulant properties. Only the thiuronium compound prepared from unsubstituted thiourea showed any significant prolongation of clotting time.

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Notes

A Note on the Isolation of Bergapten and Imperatorin from the Fruits of *Pastinaca sativa* L.*

By T. O. SOINE, HAMED ABU-SHADY, and F. E. DiGANGI

Pastinaca sativa L. (wild parsnip) has been known to cause contact dermatological reactions or percutaneous photosensitizations (1–3). Insofar as the authors are aware, no serious phytochemical investigations have been carried out on the fruits or other plant parts to determine the causative agent or agents. Coumarins have been noted by Casparis and Manella (4) as being present in the fruits of *Pastinaca sativa* but not in the roots. No effort was made by these workers to further identify the coumarins present.

In the present work the authors have isolated and characterized two furanocoumarins, namely bergapten and imperatorin, from the fruits of wild parsnip. That these agents could well be one of the causes for the photosensitization effects of this herb is illustrated by the fact that the authors were able to induce marked tanning of the skin of their forearms in all cases with imperatorin and in one case with bergapten. In the case of one of us (F. E. D.) there was a marked reaction to the application of both these materials to the extent that it could be termed a second degree burn. This reaction to furanocoumarins has been noted by Fahmy and Abu-Shady (5) wherein they showed that imperatorin, bergapten, and xanthotoxin all had activity but the latter was the most potent in this respect.

EXPERIMENTAL

Material.—The fruits of *Pastinaca sativa* L.[1] were collected when fully mature from plants growing wild in July, 1952, within the city limits of Minneapolis, Minn. They were ground to a No. 20 powder in a Jacobsen laboratory grinder.

Extraction.—The powdered fruits (500 Gm.) were placed in a Soxhlet apparatus and extracted continuously for four hours with petroleum ether (b. p. 30–60°) at which time a white solid had separated. This was removed by filtration of the hot percolate and found to weigh 1 Gm. The filtrate was diluted to one liter with petroleum ether and then placed in a deep freeze for five days during which time a crystalline precipitate formed weighing 3.1 Gm. Further concentration and cooling of the filtrate from these crystals failed to yield further significant amounts of material. The petroleum

* Received April 21, 1956, from the Department of Pharmaceutical Chemistry, College of Pharmacy, University of Minnesota, Minneapolis 14.

[1] The authors are grateful to Dr. Gerald Ownbey, Botany Department, University of Minnesota, for the identification of the plant material. A herbarium specimen has also been deposited in the archives of the Botany Department, University of Minnesota.

ether was finally completely removed from the percolate and the oil was then extracted several times with small portions of boiling methanol. These alcoholic extractions were combined and chilled to remove excess oil and finally evaporated to dryness but did not yield significant amounts of crystalline material. The crystalline material obtained from the hot percolate was recrystallized several times from 95% ethanol to yield long, white needles, m. p. 190–1°. Their behavior toward alkali suggested a coumarin nature and this, together with the melting point, indicated that the material was probably bergapten. A mixed melting point with authentic material previously obtained from *Ammi majus* L. fruits showed no depression of the melting point. This observation coupled with the analytical data conclusively identified the material as bergapten.

Anal.[2]—Calcd. for $C_{12}H_8O_4$: C, 66.67; H, 3.73. Found: C, 66.44; H, 3.68.

The mother liquors from the previous recrystallizations were combined and stripped of solvent and the residue combined with the 3.1 Gm. of crystalline material obtained by the freezing procedure.

The Soxhlet percolation was carried on for another twenty hours on the marc from the four-hour extraction with a fresh charge of petroleum ether. When this percolate was worked up in a similar manner to that described for the four-hour percolate it yielded 0.5 Gm. of solid semicrystalline material which was combined with the 3.1 Gm. of crystalline material previously referred to. This combined crystalline material was then recrystallized from alcohol to yield an additional quantity of bergapten as determined by the mixed melting point. The mother liquor was finally concentrated and allowed to stand in the deep freeze for a period of two days during which time a greenish colored crystalline material separated, weighing 1.5 Gm. This material had a melting point of 80–95°. It was crystallized twice from ether to yield 0.5 Gm. of crystals, m. p. 100–1°. This material was soluble in hot alkali to give a yellow solution; its color tests (5) and solubilities coupled with its melting point suggested that the compound was imperatorin. A mixed melting point with authentic imperatorin, previously obtained from *Ammi majus* L., showed no depression of the melting point.

Anal.—Calcd. for $C_{16}H_{14}O_4$: C, 71.10; H, 5.22. Found: C, 70.92; H, 5.06.

No further crystalline material which did not correspond to imperatorin could be isolated from the residues although we are presently carrying out chromatographic studies in an effort to identify further constituents. Another report will describe our findings.

Photosensitization Tests.—Both of these materials, namely imperatorin and bergapten, were made up into a 1% alcoholic solution and then applied to the inner part (ventral) of the forearm of each of the authors. The treated area was then exposed to sunlight in the amount that would normally be obtained during the summer months wearing short sleeved shirts. A noticeable tanning effect was apparent within a few days and lasted well into the fall, gradually fading to the normal skin color. All subjects gave a reaction of this type with imperatorin but only one (F. E. D.) reacted to bergapten. In the case of this one individual there was a very pronounced reaction with erythema, pruritus, and vesication accompanying the tanning effect.

[2] All analyses were carried out by the Microanalytical Laboratory, School of Chemistry, University of Minnesota, Minneapolis.

SUMMARY

1. Imperatorin and bergapten have been isolated from the fruits of *Pastinaca sativa* L. (wild parsnip).

2. Inasmuch as they are furanocoumarins and have been shown to exert a definite photosensitization effect on the human skin they are believed to be at least partially responsible for these effects of *P. sativa* L. noticed by other workers.

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SEROLOGY OF SYPHILIS—REFRESHER COURSES

Nine laboratory refresher courses covering the serology of syphilis will be offered at the Venereal Disease Research Laboratory in Chamblee, Ga., from August 1956 to May 1957 under the Public Health Service's Venereal Disease Program.

The schedule of the courses is as follows: Serology of Syphilis, Aug. 20–31, 1956; Sept. 17–28, 1956; Management and Control of Syphilis Serology by the Regional Laboratory, Oct. 8–19, 1956; Tests for Syphilis Using the *Treponema pallidum*, Oct. 22–Nov. 2, 1956; Serology of Syphilis, Dec. 3–14, 1956; Feb. 4–15, 1957; Mar. 18–29, 1957; Tests for Syphilis Using the *Treponema pallidum*, Apr. 8–19, 1957; Management and Control of Syphilis Serology by the Regional Laboratory, Apr. 22–May 3, 1957.

The *Serology of Syphilis* courses are designed to provide refresher training to operating personnel of State and public health service laboratories and to other qualified individuals from this and other countries.

Correspondence about these courses should be addressed to: Director, Venereal Disease Research Laboratory, Division of Special Health Services, PHS, Department of Health, Education, and Welfare, P. O. Box 185, Chamblee, Ga.

A Note on A Plastic Insert for Adapting Beckman Corex Cells to Microanalysis*

By THOMAS J. HALEY, W. G. McCORMICK, and R. W. DICKINSON†

IN THE DETERMINATION OF iron binding capacity of blood serum by the method of Schade, *et al.* (1), a final volume of solution of one ml. is obtained. To avoid the complication from excess bleeding of the experimental animals it is desirable to maintain this volume. Thus, to overcome the expense of a special microcell and the difficulties of procurement, it was necessary to convert the standard Beckman Corex cell (volume 3 ml.) into a microcell. To accomplish this a plastic insert was designed to fill the excess space.

Figure 1 shows the basic construction of the insert. Since the Corex cells are not made to exact dimensions, each insert must be especially fitted to each cell. The slit width of the spectrophotometer will determine the size of the light pathway and the volume of solution required. The light pathway through the insert must be at least one mm. larger in diameter than the light path so that all the light goes through the test solution and does not strike the insert.

To find the size of the light path, remove the phototube housing and the cell compartment; turn on the instrument and adjust the slit width to the desired opening. Place a piece of paper over the opening and trace the outline of the light on the paper. Replace the cell compartment and check the size of the light path again. The tracing with an additional one mm. in diameter margin gives the dimensions of the light pathway. As a final precaution, check the density readings of distilled water in the cells with and without the inserts.

These plastic inserts can be made from Lucite or Teflon. The latter material is recommended for those instances in which corrosive chemicals or solvents that would react with Lucite are being analysed.

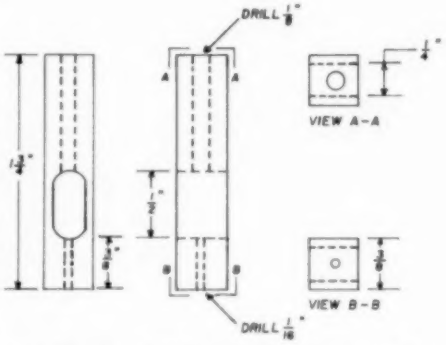

Fig. 1.—Plastic insert for Beckman spectrophotometer cell.

These inserts are easy to construct and cost very little. Furthermore, they allow a wider use to be made of existing equipment.

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(1) Schade, A. L., Cyama, J., Reinhart, R. W., and Miller, J. R., *Proc. Soc. Exptl. Biol. Med.*, **87**, 443(1954).

* Received December 5, 1955, from University of California, Atomic Energy Project, Los Angeles.
This article is based on work performed under Contract No. AT-04-1-GEN-12 between the Atomic Energy Commission and the University of California at Los Angeles.
† Division of Pharmacology and Toxicology, Atomic Energy Project, School of Medicine, University of California at Los Angeles.

A Note on the Metabolism of Lysergic Acid by *Claviceps Purpurea* (Fries) Tulasne*

By GUNNAR GJERSTAD

IN RECENT YEARS considerable interest has centered around the problem of making the ascomycete, *Claviceps purpurea* (Fries) Tul. produce the medicinally important ergot alkaloids when grown in synthetic or semisynthetic substrates (1). There seems to be but little agreement among the investigators on the anabolitic power of *Claviceps* species. Occasional reports that alkaloids were extracted from artificial media have proved to be nonreproducible.[1]

A few of the published works (2–4) claim that ergot alkaloids when added to the media are metabolized quickly by the fungus. Also, Abe (4) in a more recent work states that "the ergot alkaloids produced in the mycelium during the culturing process are gradually consumed by the fungus."

* Received December 12, 1955, from University of Texas, College of Pharmacy, Austin 12.

[1] Private communication from Dr. G. Shahovskoy, formerly with Eli Lilly and Co., Indianapolis, Ind.

In a previous work (5) we were not able to reproduce this finding, and it does not seem too logical that alkaloids produced during parasitic ergot development and exposed to all types of climatic conditions should be less subject to deterioration. Michener and Snell (3) reported that alkaloids disappeared from noninoculated media as well.

Since it is generally and logically assumed that *d*-lysergic acid, an integral part of all ergot alkaloids, is an intermediate in their biogenesis, we were interested in determining whether or not this acid is converted into the known ergot alkaloids when fed to *Claviceps* in artificial media. As basic substrate the following medium was used: magnesium sulfate 1 Gm., potassium dihydrogen phosphate 2 Gm., peptone 6 Gm., maltose 50 Gm., sucrose 10 Gm., asparagine 1 Gm., and water to make 1 L.

To this medium at a *p*H of 5.6 was added *d*-lysergic acid[2] to make a saturated solution. The quantity which could be dissolved at this *p*H amounted to 1 mg. per 10 cc. (10 mg. %).

After the medium had been sterilized by ultrafiltration, an authentic conidioculture of *Claviceps purpurea* of a high alkaloid producing capacity was inoculated into four 10-cc. portions of the substrate. A fifth batch was kept as blank control, since Michener, *et al* (3), reported that ergot alkaloids disappear rapidly from noninoculated media as well, and it was of interest to test if the same would happen to lysergic acid.

A 33-day growth period without shaking was allowed for full development. Fungus growth was prolific, and yielded a larger quantity of air dried fungal material than ordinary basic media (5). This indicates strongly that if lysergic acid is an intermediate compound, the pathways of alkaloid formation in ergot may not be, as frequently claimed, the result of a detoxification reaction.

The growth results may be tabulated as in Table I.

TABLE I

Sample No.	Appearance	*p*H After Termination of Growth	Wt. of Air Dried Fungal Material in mg.
1	White thick pellicle with few large conidia	6.0	244
2	Grayish aerophilic pellicle; more conidia than sample 1	5.8	298
3	Gray pellicle; abundant conidiospores	6.1	343
4	Grayish-green, folded pellicle. No conidiospores	5.5	204

From Table I it appears that polymorphism among identical cultures is predominant, a finding which is unexplained, but which has been reported by other investigators in the field (1, 5).

It appears that the higher the final *p*H of the medium, the greater the yield of fungal material and the more abundant is the conidiospore formation.

[2] Sample furnished through courtesy of Sandoz Pharmaceuticals, Inc., of Rahway, N. J.

The media, including the blank, turned slightly blue during the duration of this experiment, possibly due to a deterioration of the lysergic acid.

After the termination of the growth experiments, the media were subjected to a quantitative analysis with respect to contents of the lysergic acid type compounds, as evaluated by the blue color produced with *p*-dimethylaminobenzaldehyde, T. S., U. S. P. One part of medium, suitably diluted to render a transmittance of above 60%, was mixed with 2 parts of the above reagent, allowed to stand for thirty minutes, and absorption measured in a Beckman spectrophotometer, model DU, at a wavelength of 575 mμ at which the highest absorption was obtained. This wavelength is slightly higher than that given in the U. S. P. XIV. Strong and Maurina (6) incidentally suggested that a wavelength of 590 mμ be used.

It was established that the transmittance among three of the culture media and the blank varied ±3.7%, the remaining parallel showing 8.2% higher transmittance than the blank. This apparently larger quantity of lysergic acid in the culture filtrate is accounted for by a larger evaporation in this particular batch during the rather long cultivation period.

In order to verify that the compound producing the blue color with the reagent was lysergic acid, all the culture filtrates were subjected to paper partition chromatography. Descending formation was used and Whatman filter paper No. 1. Two different solvent mixtures were employed: (*a*) *n*-butanol, acetic acid, water (4 + 1 + 5 by volume) and (*b*) *n*-butanol, pyridine, and water (4 + 1 + 5 by volume). After drying the papers were inspected under ultraviolet light for characteristic blue fluorescence.

R_f values for characteristic spots were for solvent No. 1, 0.66 and for solvent No. 2, 0.21, which were identical with the R_f values exhibited by control runs of authentic *d*-lysergic acid. No other spot was discernible within the concentrations used, indicating that none of the reaction products with the *p*-dimethylaminobenzaldehyde reagent was due to formed ergot alkaloids.

The lower limit for detection of ergonovine on a chromatographic sheet was determined to 0.02 μg. in 5 λ; a quantity of 0.015 μg. of lysergic acid could be detected.

Both the above compounds are detectable with v. Urk's reagent in a concentration of 0.0001%; the actual, absolute quantity is dependent on the size of the employed cuvettes. Since these quantities are approximately 1/1000 of the amount of alkaloids usually found in ergot, we might consider a negative test as an indication that no lysergic acid type compound is present.

CONCLUSION

1. Lysergic acid when added to synthetic substrates, is not metabolized by *Claviceps*, and the question arises whether this compound actually constitutes an intermediate in the biosynthesis of ergot alkaloids.

2. The minimal concentrations for the detection of *d*-lysergic acid and *l*-ergonovine according to two current analytical methods are reported.

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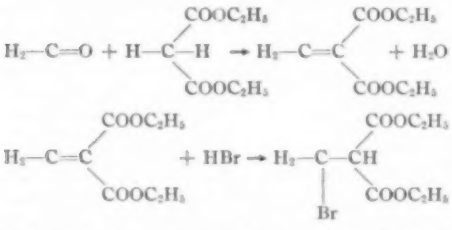
A Note on a New Method of Preparation of (Bromomethyl) Malonic Ester

By JOSEPH G. CANNON*

THE ONLY METHOD reported in the literature for the preparation of (bromomethyl) malonic ester has been that of Simonsen (1) in which monochloro dimethyl ether is condensed with sodio malonic ester to give (methoxymethyl) malonic ester, which is subjected to HBr cleavage of the ether link to form (bromomethyl) malonic ester. Yields in the two steps were 52.5% and almost quantitative, respectively.

A more direct method of synthesis is proposed:



This method produces a final product which is considerably purer than that prepared by Simonsen's method, and in addition, the time of preparation is reduced markedly, although the yield is somewhat lower.

EXPERIMENTAL

Diethylmethylenemalonate.—The method of Bachman and Tanner (2) was used with some modification. Two hundred grams of glacial acetic acid, 30 Gm. of paraformaldehyde, 80 Gm. of malonic ester, 5 Gm. of anhydrous potassium acetate and 5 Gm. of anhydrous cupric acetate[1] were mixed in a 500-ml. two-neck flask equipped with a stirrer and a condenser fitted with a calcium chloride tube. The mixture was heated for three hours in an oil bath kept at a temperature of 120–125°. The reaction mixture was then distilled; the fraction distilling between 140° and 200° (25 mm.) was collected and redistilled through a column. The fraction distilling at 205–207° (750 mm.) was methylene malonic ester. Yield, 23 Gm. (40%).

(Bromomethyl) Malonic Ester.—Under anhydrous conditions, 15 Gm. of diethyl methylene malonate was dissolved in a threefold volume of ether. The flask containing the ethereal solution was then immersed in an ice water bath, and anhydrous hydrogen bromide was bubbled slowly through the solution until the gas was no longer absorbed (about an hour). The solution was then poured over ice, and solid sodium bicarbonate was added in small amounts until the solution was alkaline to litmus. The entire mixture was then transferred to a separatory funnel, and the water layer was drawn off. The ethereal layer was washed twice with ice cold water, the washings being added to the aqueous layer. The aqueous portion was re-extracted with ether; the ethereal layer was then combined with the original ether extract. After the ether extract was dried over anhydrous magnesium sulfate, the ether was removed on a steam bath. The residue was distilled from an oil bath, omitting a condenser from the apparatus, and condensing and collecting the distillate directly in a receiver immersed in a dry ice-acetone bath. B. p. 95–97° (1 mm.). Yield, 15 Gm. (68%, based on methylene malonic ester). n_D^{20} 1.4501; sp. gr. (20°) 1.337.

The (bromomethyl) malonic ester was analyzed for bromine by a Volhard procedure, after refluxing the weighed sample with 10% potassium hydroxide in alcohol for twenty minutes, then acidifying with nitric acid.

Anal.—Calcd. for $C_9H_{13}O_4Br$: Br, 31.57. Found: Br, 28.98, 29.19, corresponding to a (bromomethyl) malonic ester content of 92%. A product prepared by the method of Simonsen was found by Leete and Marion (3) to contain 26.8% bromine, corresponding to a (bromomethyl) malonic ester content of 85%.

In addition, the molecular weight of the compound was determined by means of its depression of the freezing point of benzene.

Anal.—Calcd. for $C_9H_{13}O_4Br$: 253.1. Found: 253.9.

* Received November 23, 1955, from the College of Pharmacy, University of Illinois, Chicago 12.

[1] Prepared from the monohydrated salt by refluxing it with a large excess of acetic anhydride for six hours.

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A Note on Pressor Drugs. VI. Acute Toxicity of Epinephrines*

By JAMES C. MUNCH†

AN INVESTIGATION was undertaken to determine the toxicity of *l*-, *d*-, and *r*-epinephrines of known purity. Data obtained in determining the quantities required to kill half of the test animals within twenty-four hours after administration are recorded in Table I. With certain tests no deaths developed. These are indicated by the table footnote reference, which denotes the largest dose that could be tested under experimental conditions. Investigations on chronic toxicity are under way and will be reported subsequently.

TABLE I.—ACUTE TOXICITIES OF EPINEPHRINES: LD_{50} mg./Kg.

	Method of Administration											
	Oral		Subcut.			I.V.			I.P.		Inhalation	
Animal	*l*	*r*	*l*	*d*	*r*	*l*	*d*	*r*	*l*	*r*	*l*	*r*
Mouse	50	90	1.5	22.5	16	...	...	...	4	7.5	...	15[a]
Rat	30	...	10	100	16	0.04	0.80	0.07	0.9	1.25	15[a]	15[a]
Guinea pig	...	...	1	...	..	0.2	...	...	2	...	15[a]	15[a]
Rabbit	30	70[a]	10	7	40	0.2	0.4	0.3	...	...	15[a]	15[a]
Cat	...	...	20	...	..	0.7	...	...	...	...	...	15[a]
Dog	...	...	5	...	..	0.3	...	...	0.7	...	...	...
Man	0.6[a]	...	1	...	..	0.01[a]	...	...	0.1[a]	...	5[a]	5[a]

[a] Denoted values which did not kill.

It was found that the determination of a numerical value for the LD_{50} was difficult, since it is altered by differences in sex, body weight, diet, method of housing, method of caging injected animals during observation, method of administration, *p*H, type and quantity of preservative, and the state of development of the nervous system. These results tended to confirm a literature report (1) upon the toxicity of *l*-epinephrine in a constant dose of 0.14 mg., intravenously injected to rats weighing between 110 and 430 Gm.; death developed in 27% of the females and 78% of the males. The addition of 0.2% of sodium bisulfite (in itself innocuous) more than doubled the acute toxicity of *l*-epinephrine to mice, rats, rabbits, and dogs following subcutaneous or intramuscular injections, without altering significantly the intravenous toxicity (8).

Reported values of effective and toxic doses on man showed even greater variations than for animals. The minimal quantity of *l*-epinephrine constantly injected intravenously to produce a slight pressor response ranged from 0.05 γ/Kg./minute for man, to 0.5 γ for rabbits, cats, and dogs, and 1 γ for rats (3). Intravenous injection of 10 γ of *l*-epinephrine produced a greater pressor response in neurotic and in psychotic patients than in controls (6).

Contrary to general opinion, Brems (2) observed therapeutic responses after the oral administration of 4 cc. of 1% *l*-epinephrine solution (equivalent to 40 mg.). After intramuscular injections of 7 mg. of *l*-epinephrine (7), or 17.5 mg. intravenously (4) recovery has been noted. Very few deaths have developed following the injection of millions of doses of epinephrine solution in dental practice. Only 6 deaths have been reported in the literature following subcutaneous injection of *l*-epinephrine (5), and none after *d*-epinephrine or *r*-epinephrine. Of these 6 deaths 3 followed subcutaneous administration of 0.6 cc. of 10% *l*-epinephrine inhalant solution by mistake for 0.1% injectable solution. No deaths have been found following inhalation of *l*-, *d*-, or *r*-epinephrine solutions, nor could any be produced in test animals following inhalation from a standard Vaponefrin nebulizer. This may be associated with other evidence that material of proper particle size is not absorbed following inhalation therapy.

* Received April 11, 1955, from the Vaponefrin Company, Upper Darby, Pa.
Presented to the Scientific Section, A. PH. A., Miami Beach meeting, May, 1955.

† Medical Director, Vaponefrin Company, Upper Darby, Pa.

SUMMARY

Data have been obtained on *l*-epinephrine, *d*-epinephrine, and *r*-epinephrine of known purity, following various methods of administration to groups of animals of similar susceptibility and under similar test conditions. The median lethal doses are given in the table, for production of death within twenty-four hours. The toxicity of *l*-epinephrine ranges from 2 to 20 times that of *d*-epinephrine, and *r*-epinephrine shows intermediate values. The toxic doses are some five hundred times the therapeutic doses. No deaths were produced by inhalation of nebulized epinephrines.

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A Note on an Indwelling Cannula for Intraventricular Injection of Drugs in the Unanesthetized Dog*

By THOMAS J. HALEY and R. W. DICKINSON

FELDBERG AND SHERWOOD (1) have described a permanent cannula for the intraventricular injection of drugs in cats. This cannula was screwed into the skull and its tip extended into the lateral ventricle. The thinness of the bone often caused the cannula to become loose and fall out. In our work with dogs (2–5) we too encountered similar difficulties with cannulas which were screwed into the skull. In the cannula described below, the possibility of loosening and falling out has been eliminated by attaching it to the skull with two screws (Fig. 1). This arrangement allows the cannula to be supported in three places and braced between the points of attachment with a thin flange of stainless steel.

The cannula is constructed from an 18-gauge, B-D Yale hypodermic needle and stainless steel bar stock according to the design shown in Fig. 1. Detail A is machined from stainless steel bar, tapped to suit the hub thread, and knurled on the outside. Detail B is a rubber disk cut from any sterile vial, self-sealing diaphragm. Detail C shows a standard B-D Yale hypodermic needle which has been reworked by removing the upper part of the hub down to the "square" and machining the remaining hub to $^1/_4$ inch outside diameter and threading 32 tpi. The inside is not altered. Detail D is of 26-gauge (0.019) stainless steel bent around a 2-inch diameter bar; spring-back leaves this attachment plate with approximately $1^3/_4$ inch radius which was suitable for our particular purpose. In our earlier units a little trouble had been experienced by the solder breaking away; later units have a reinforcing disk sweated to the attachment plate and both of them are heavily soldered on the upper side to the tip of the hub. This also prevents the cannula, which is usually swaged into the hub, from working loose. Detail E is a needle stylet bent at the end so as to rest on top of the hub but not so long as to interfere with the cap being screwed into place.

The great variation in head size of dogs makes it necessary to vary the length of the cannula. Cannula lengths of 29, 33, or 35 mm. will penetrate into the third cerebral ventricle of most dogs and the appropriate length is chosen at the time of implantation.

The dog is anesthetized with 35 mg./Kg. of pentobarbital, intraperitoneally, and the cannula is implanted. A 40-mm. lateral incision is made in the skin of the head at a 45° angle with the midline, 20 mm. anterior to the base of the ear. With the skin retracted a similar incision is made through the fascial layer to expose the muscle. The muscle is divided and retracted to expose the skull. Keep bleeding to a minimum. Drill a $^1/_{16}$-inch hole in the skull and insert the cannula containing the stylet. Cerebrospinal fluid should well up in the cannula if the third ventricle has been entered. Drill two holes in the skull and secure the cannula with No. 2 round head wood screws. Close the wound and give the animal 100,000 units of repository penicillin, intramuscularly. Eight to ten days later the animal can be used for injections, which should be kept below 1 ml. in volume. Dogs with such implanted cannulas have been kept for months without any apparent ill effects from the cannula.

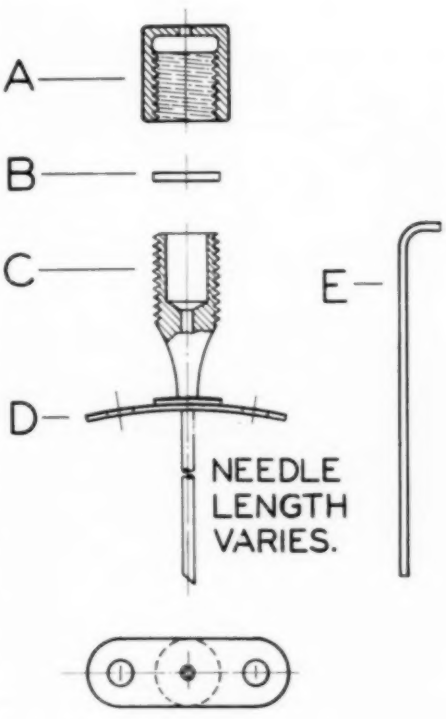

Fig. 1.—Details of indwelling cannula.

* Received December 19, 1955, from the Division of Pharmacology and Toxicology, Atomic Energy Project, School of Medicine, University of California at Los Angeles.
This article is based on work performed under Contract No. AT-04-1-GEN-12 between the Atomic Energy Commission and the University of California at Los Angeles.

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A Note on Sabadilla Alkaloids. Cevadine*

By SAMUEL J. RINGEL

BECAUSE OF interest in the insecticidal properties of sabadilla alkaloids, a study of procedures for their extraction was begun. Initially a modification of the procedure of Poetsch, *et al.* (1, 2), was followed, using Skellysolve B as the solvent. Then, for purposes of comparison of yields of crude alkaloid obtained with various other solvents, Skellysolve A, C, D, E and, lastly, chloroform were used in extraction experiments on one lot of sabadilla seed. Preliminary experiments on chromatographic separation of sabadilla alkaloids were performed. After reaching the stage of comparison of yields of alkaloid obtained with the solvents mentioned above and the preliminary findings in chromatography, it was found necessary to suspend work on this project, but the findings, although fragmentary, may be of interest to those currently engaged in research on sabadilla alkaloids.

The percentages of crude alkaloid extracted from sabadilla seed by the solvents used are as follows: Skellysolve A—2.03, 2.02; B—0.93, 0.93; C—1.80, 1.83; D—2.07; E—2.63; chloroform—3.20. Ikawa, *et al.* (3) found that Skellysolve A extracted only an insignificant amount of alkaloidal material. On drying the crude alkaloid *in vacuo*, the higher temperature and longer drying time necessary for the Skellysolve D and E extracts resulted (markedly so in the case of the latter) in decomposition of the material, so that there would appear to be no advantage in using petroleum ether solvents boiling higher than Skellysolve C for initial extraction of the alkaloidal mixture.

In further processing the crude material to obtain cevadine the following percentages were extracted by the solvents used: Skellysolve A—3.28; B—24.47; C—32.89; D—45.00; and E—28.85.

The higher melting alkaloid fractions (195° and above) obtained from the petroleum ether extracts at this stage were combined and purified by crystallizing from 50 per cent ethanol. The melting point of analytically pure cevadine thus obtained was 214–215°. This coincides with the value given by Pelletier and Jacobs (4). Derivatives of cevadine were prepared, analyses run to confirm their purity, and melting points recorded (Table I).

TABLE I

Derivative	M. p. found °C.	In literature °C.
Mercuric chloride	190 (dec.)	172 (5)
Auric chloride	200 (dec.)	182 (5), 190 (3)
Diliturate	230	220 (3), 222 (2)

The infrared spectrum of cevadine showed absorption attributable to the carbonyl group in the ester linkage and to the multiplicity of hydroxyl groups, but no other distinguishing features. Comparison with a sample of cevadine furnished by Stoll (6) confirmed the identity of the compound isolated by the author.

In the preliminary chromatographic work the crude alkaloid mixture was eluted from a silicic acid column, using an isooctane-chloroform mixture as the eluting medium. The progress of the bands through the column, which contained a fluorescent material, was followed by means of an ultraviolet lamp. Several fractions giving the typical ultraviolet spectrum of veratridine were obtained.

After preliminary screening by Maison (7) cevadine was tested as a hypotensive agent by Freis who stated in a personal communication that the hypotensive dose in man was 50–60 mcg./Kg., and that the undesirable side effects associated with veratrum alkaloids were also present when cevadine was used.

* Received October 14, 1955, from the Entomology Research Branch, Agricultural Research Service, United States Department of Agriculture, Beltsville, Md.

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(7) Maison, G. L., Gotz, E., and Stutzman, J. W., *J. Pharmacol. and Exptl. Therap.*, **103**, 74(1951).

FIFTH ANNUAL GLYCERINE RESEARCH AWARDS ANNOUNCED

The Glycerine Producers' Association has announced that the fifth annual Glycerine Research Awards are now open and that nomination blanks are available. This announcement was contained in a new bulletin describing the purpose of the awards, work eligible for consideration, method of nomination and judging, dates by which nominations must be received, and previous winners.

First award is $1000 and an honor plaque, second award $300, and third award $200. These awards are granted for independent research leading to new and improved applications of glycerine or glycerine derivatives.

Copies of the bulletin giving complete details on the awards may be obtained from the Glycerine Producers' Association, 295 Madison Avenue, New York 17, N. Y.

Book Notices

Pharmacopoea Internationalis. 1st ed. Vol. 2. World Health Organization, Geneva, 1955. xx + 350 pp. 16.5 x 24.7 cm.

Volume II of the first edition of the International Pharmacopoeia adds to the standards of Volume I which was published in 1951 and was reviewed in THIS JOURNAL, **41,** 222(1952). The new volume includes newer basic drugs such as chlortetracycline hydrochloride, choline chloride, cyanocobalamin, cortisone, dihydrostreptomycin and streptomycin, etc. Older drugs for which standards are now provided include chlorobutanol, bismuth subnitrate, benzylpenicillin potassium, etc. The major difference between the two volumes is the inclusion in Volume II of many drugs in dosage forms as tablets and injections. Appendices present additional tests and analytical methods required for the new monographs. A table of usual and maximal doses for adults of drugs in Volume II and a table of usual daily doses for children of certain drugs in Volumes I and II are included.

Tables comparing drug titles and strength standards of drugs appearing in both the Ph. I. Volumes I and II and in N. F. X are included in the General Information section of N. F. X.

Epitome of the Pharmacopeia of the United States and the National Formulary with Comments. 10th ed. Issued under the direction and supervision of the Council on Pharmacy and Chemistry of the American Medical Association. J. P. Lippincott Company, Philadelphia, 1955. xiii + 322 pp. 13 x 19.2 cm. Price $3.

This edition (10th) of the Epitome follows the general plan of previous editions and includes drugs in U. S. P. XV and N. F. X. The actual text, however, is designed to serve as a companion volume to *New and Nonofficial Remedies,* giving more detailed information regarding actions and uses of drugs not in the current N. N. R. Some attempt at therapeutic evaluation of official drugs is made, as in the case of lobelia N. F.: "Obsolete, ineffective, expectorant, nauseant, and emetic, resembling nicotine;" and fennel oil U. S. P.: "Aromatic carminative little used today." It is a handy pocket-size reference volume for the physician.

Product Liability Cases. By Frank T. Dierson and Charles Wesley Dunn. Commerce Clearing House, Inc., Chicago, 1955. xlvii + 1134 pp. 17.5 x 25.2 cm. Price $12.

This book which is the eighth research volume in the "Food Law Institute Series" compiles the modern and leading cases in the area of the product liability law, relating to foods and drugs and cosmetics. The cases are headnoted, and they are annotated by reference tables and an index. The cases are also listed at the front of the book in alphabetical order and by jurisdictional classification. Most of the text (819 pages) is devoted to significant product liability cases that have been brought to the courts and completed during the years 1947–1953. Additional significant cases, regardless of time of contest, are presented in the second part (294 pages) of the book. The book is an essential reference for individuals or groups concerned with the covered phase of legal liability.

Methoden der organischen Chemie. Vol. 3, Part 1, Physikalische Methoden. (Houben - Weyl). Georg Thieme Verlag, Stuttgart, 1955. xxix + 954 pp. 18.5 x 26 cm. Price DM 162.

This is the seventh volume in the new series of *Houben-Weyl* and, although it is Part 1 of Volume 3 its publication follows the appearance of Part 2 of Volume 3 which was reviewed in THIS JOURNAL, **45,** 127(1956). Part 1 is devoted, as is Part 2, to the methodology of physical research applied to preparative organic chemistry.

The text is divided into sixteen chapters, each prepared by a specialist in the field. The first chapter is concerned with thermodynamics which is presented in the manner followed at the University of Tübingen. The second chapter describes methods of conducting kinetic experiments including reactions important in macromolecular chemistry. The following chapters cover determinations of density, solubility, vapor pressure, and methods for the determination of molecular weights, the latter being divided into chapters on lower molecular weight compounds and on heavier molecules. A short chapter on surface tension and activity is followed by a more comprehensive treatment of calorimetric methods. A chapter is devoted to stereochemical models of atoms and molecules. A summary of mechanical and thermal methods includes a section on the errors in physical measurement. The extensive descriptions of microscopic methods are considered to be important to the organic preparative chemist, and the detailed coverage of crystallographic methods and apparatus can be particularly useful in the field of identification requirements that is gaining increased recognition because of its specificity. The final chapters in this volume concern mass spectrometry and methods of working with radioactive and stable isotopes, and the material in this volume complements that in the chapter on "Preparation of Isotope Labeled Compounds" in Volume 4, Part 2, of this treatise. The contributors, editors, and printers have done an outstanding job in the preparation and publication of this volume and the other volumes that have appeared.

The references and documentation in this standard reference work brings the text material up to date; in some cases literature of 1955 is cited. The tables of abbreviations for literature cited and for technical terms at the front of the book and the thorough author and subject indexes appended continue the style set in the earlier volumes. Reference libraries will want this volume and will hopefully await the early appearance of the other volumes in the series.

The Systematic Identification of Organic Compounds. 4th ed. By Ralph L. Shriner, Reynold C. Fuson, and David Y. Curtin. John Wiley & Sons, Inc., New York, 1956. ix + 426 pp. 14.5 x 22 cm. Price $6.

The fourth edition of this laboratory manual enables the student to observe the relation between functional groups and chemical behavior in identification tests and reactions. Physical characteristics, such as melting points and mixed melting points to distinguish between starting and final products, are stressed. A chapter on the rise of spectroscopic methods for functional group determination is included, but the full application of ultraviolet and infrared procedures for identification of compounds is intentionally limited. Tables of properties of organic compounds and their derivatives are included. This useful manual merits the consideration of teachers of organic chemistry as well as the courses limited to analytical and identification procedures.

Blood Group Substances—Their Chemistry and Immunochemistry. By Elvin A. Kabat. Academic Press, Inc., New York, 1956. ix + 33 pp. 16 × 23.6 cm. Price $8.

This book attempts to bring together in a concise manner the present information on the blood group substances as seen from the viewpoint of quantitative immunochemistry. It is hoped that the immunochemical approach will make it easier for workers and students in this field to keep up with future developments. The blood group system is used as a model for many types of immunochemical studies. The text material is divided under the headings: the human blood group factors; methods and reagents used in testing for blood group antibodies and antigens; sources of blood group substances; purification of blood group substances; chemical composition and properties of the blood group A, B, O (H) and Le^a preparations; chemical and immunochemical characterization of the blood group substances—evidence of purity; immunochemical similarities and differences among blood group substances from various species; some features of the structure of the blood group A, B, O (H) and Le^a substances; antibodies to blood group substances and their biological effects; and biological effects and uses of blood group substances—materials of similar chemical composition.

Pharmacists will be interested particularly in portions of the text relating to passage of antibodies through the placenta, relation of blood group substances to intrinsic factor and to materials inhibiting the action of various viruses. The references for each chapter and the subject index add to the value of this book which should be available to those in the medical care sciences.

Handbook of Medical Library Practice. 2nd ed. Edited by JANET DOE and MARY LOUISE MARSHALL. American Library Association, Chicago, 1956. xv + 601 pp. 16 x 24.5 cm. Price $10.

This manual of procedure for the medical library is essential for every medical and dental library, and desirable for medical historians and scholarly reference departments. Its scope is wide and its section on medical literature is useful to physicians, as well as librarians.

A revision of the first edition published in 1943, this *Handbook* is a noteworthy improvement over the original work. More attention has been given to the needs of libraries in the allied fields of dentistry, pharmacy, and nursing than in the first edition. The primary purpose of the manual is to present as simply as possible information useful to those who deal with medical literature.

Three new chapters have been added on: the Medical Library Association, photoduplication, and public relations; and new sections on book preservation and other subjects are included. The bibliography has been doubled in length. Anyone interested in the history of medicine will find this bibliography exceedingly helpful in locating material and building an historical reference collection. The virtues of this book are obvious to all who examine it, and credit should be given to its self-sacrificing compilers. It is a tremendous undertaking.

The only place for criticism of this *Handbook* is in the chapter on "Acquisition and Preservation," where names of agents are recommended for the handling of foreign subscriptions and for purchasing back issues of journals. A number of these agents could well have been omitted from the lists. Medical acquisition librarians having contact with these agents know from experience that some do not perform satisfactorily.

Despite this minute criticism, the *Handbook of Medical Library Practice* deserves representation on the reference shelf of every medical library.

Reports on the Progress of Applied Chemistry. Vol. 39. Society of Chemical Industry. Interscience Publishers, Inc., New York, 1954. 1125 pp. 14 x 22 cm. Price $6.50.

The 1954 volume of this book follows the same design in form and content as that of Vol. 38, which was described in THIS JOURNAL, 44, 128(1955). It includes sections on advances in inorganic chemistry; metals; fuel and fuel products; organic chemistry; biological products; textiles, plastics, adhesives and paints; food and agriculture; and chemical engineering and hazards. A name index and a very adequate subject index are provided and the book

is thoroughly documented with references to the significant publications during 1954. Each of the main divisions is divided into several chapters and those of principal pharmaceutical interest include fine chemicals and medicinal substances; intermediates and dyestuffs; antibiotics; essential oils; general microbiological processes; and enzymes. There is another interesting chapter on Toxicological hazards which summarizes recent advances in the recognition and prevention of risks to which the industrial worker is subjected.

The Mathematics of Physics and Chemistry. 2nd ed. By HENRY MARGENAU and GEORGE M. MURPHY. D. Van Nostrand Company, Inc., 1956. xii + 604 pp. 16 x 23.5 cm. Price $7.95.

This book is offered as a bridge for the gap between ordinary training in differential and integral calculus and the mathematical knowledge needed for new scientific developments. It is particularly suited for general use in applied mathematics as well as for thermodynamics, statistical mechanics, and quantum mechanics. With partial differentiation as used in thermodynamics as a basis, the book develops methods of solving ordinary and partial differential equations; vector analysis; the calculus of variations; eigenvalues and eigenfunctions; matrices; quantum mechanics and statistical mechanics. The text material is divided under the following chapter headings: the mathematics of thermodynamics; ordinary differential equations; special functions; vector analysis; coordinate systems; vectors and curvilinear coordinates; calculus of variations; partial differential equations of classical physics; eigenvalues and eigenfunctions; mechanics of molecules; matrices and matrix algebra; quantum mechanics; statistical mechanics; numerical calculations; linear integral equations; and group theory.

References are given along with the text and suggested additional texts are listed after each chapter. An essential index is appended. The type and printing in this book are excellent and the many formulas are easily read.

Klinik und Therapie der Vergiftungen. By SVEN MOESCHLIN. Georg Thieme Verlag, Stuttgart, 1956. Intercontinental Medical Book Corporation, New York. xi + 521 pp. 18.5 x 25 cm. Price DM 59. Price $14.05.

This book is designed for professional toxicologists, physicians, and others concerned with hazards arising in the use of household chemicals and drugs and those met with by workers in the chemical and allied industries. The content of the book is adequate for its intended purpose and the subject matter appears to be arranged in a logical order. It begins with discussions of the frequency and distribution of poisoning, general principles employed in the treatment of poisoning, and a listing of the more important materials and preparations used in the treatment of cases of poisoning. These general chapters are followed by a useful table of maximum allowable concentrations, expressed in parts per million, of gases, vapors, and dusts in the air to which workers may be exposed without creating a health hazard through inhalation of the potential poisons listed. Proceeding from this table the book is divided into sections, each with several subdivisions, relating to inorganic chemicals, organic chemicals, vitamins (AB&D), hormones, poisons from plant sources and the derivatives, antibiotics, food poisons, and animal poisons. Sources of exposure are then discussed.

The book is thoroughly documented throughout, and is provided with an author index and an unusually complete subject index. The paper, printing, and binding are of excellent quality representative of all books produced by the Georg Thieme Verlag. The author, Dr. Sven Moeschlin, of the University of Zurich, is to be commended for having done a thorough job of treating an important subject. The book can be recommended to those who are concerned with the diagnosis and treatment of cases of poisoning.

BOOKS RECEIVED

Proceedings of the Ninth Annual Conference on the Administration of Research. New York University Press, 1956. 107 pp. 21.5 x 28 cm. Price $4.00.

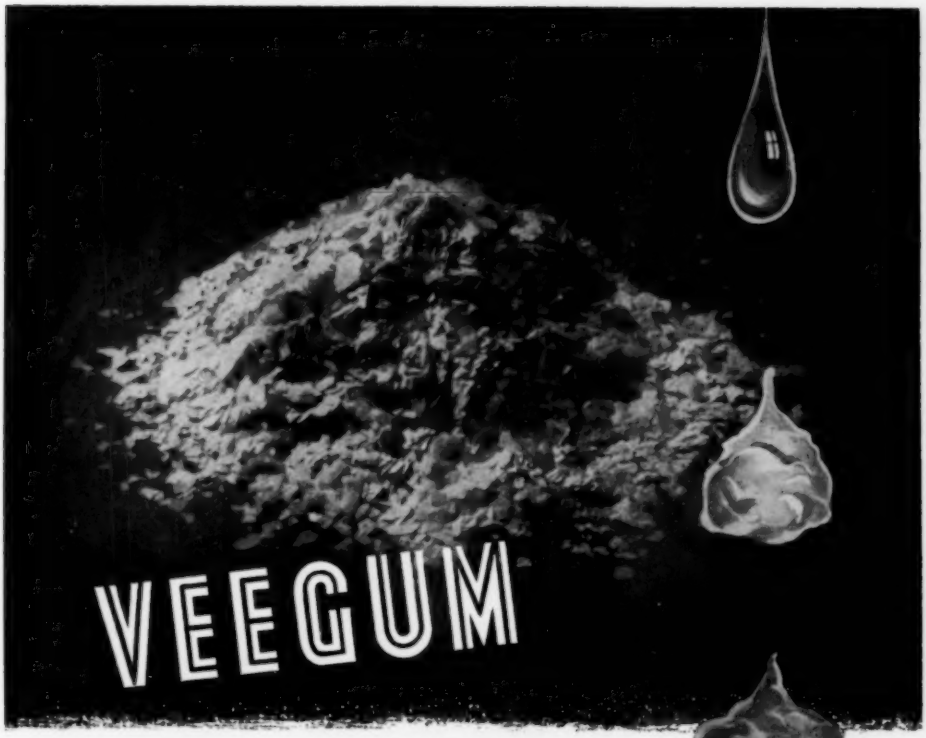
Therapy of Fungus Diseases. Edited by THOMAS H. STERNBERG and VICTOR D. NEWCOVER. Little, Brown and Company, Boston, 1955. xxiii + 337 pp. 15.5 x 24.5 cm. Price $7.50.

Methods of Biochemical Analysis. Vol. 3. Edited by David Glick. Interscience Publishers, Inc., New York, 1956. x + 437 pp. 16 x 23.75 cm. Price $9.50.

Mechanism of Organic Chemical Reactions. By E. DE BARRY BARNETT. Interscience Publishers, Inc., New York, 1956. vi + 289 pp. 14 x 22.5 cm. Price $4.75.

Synthetic Drugs. By H. RONALD FLECK. Elsevier Press, Inc., New York, 1955. viii + 380. 15 x 5 x 26 cm. Price $12.50.

Essays in Biochemistry. Edited by SAMUEL GRAFF. John Wiley & Sons, Inc., New York, 1956. x + 345 pp. 14.5 x 23.5 cm. Price $6.50.



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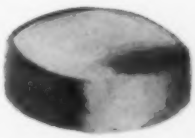

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